University of Alberta

Furthering Characterization of Human HERC5: A Novel Member of the Antiviral Response

by

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Abstract

HERC5 was initially characterized as a protein capable of binding to cyclins, a family of cell cycle-associated proteins. Further investigations into the transcriptional regulation of HERC5 demonstrate that it is responsive to viral infection and oncogenic transformation. Research presented here provides direct evidence that HERC5 is an interferon-responsive gene, and is in fact directly transcriptionally activated following stimulation of the type I interferon receptor. HERC5 is also stimulated by a double-stranded RNA responsive pathway, and while this activation displays delayed kinetics, it also demonstrates a much greater transcriptional induction. Furthermore, findings presented here draw into question the role of HERC5's putative interaction with cyclins, as their localization appears to be independent of one another. Finally, a role for HERC5 in modulating the cytoskeleton and/or cell cycle factors is proposed, subsequent to demonstrating that ectopic expression of HERC5 in U-2 OS cells induces nuclear dysmorphisms reminiscent of mitotic catastrophe.

Dedication

My years at the University of Alberta have brought me many opportunities, experiences, and immense happiness. The constant love and support of my family helped me to weather the hard times and to appreciate and celebrate every success. I would like to acknowledge my grandparents: Bill Quest, Marge Quest, and Mae Schulz, my parents-in-law: Marek and Hanna Michalak, my parents: Dale and Debra Quest, and my brother: Braden Quest, for their infinite love and support. Finally, to my wonderful wife, Karolina: you are my sunshine, joy, and love every day of my life.

This thesis is dedicated to my family.

Your love and support means the world to me in everything I do.

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List of Abbreviations

APAF-1: Apoptosis Activating Factor 1

APC/C: Anaphase Promoting Complex/Cyclosome

ATF-2: Activating Transcription Factor 2

ATM: Ataxia Telangiectasia Mutated

ATP: Adenosine Triphosphate

ATR: Ataxia Telangiectasia and Rad3 Related

BSA: Bovine Serum Albumin

BH: BCL-2 Homology

CAD: Caspase Activated Deoxyribonuclease

CDK: Cyclin-Dependent Kinase

cDNA: Complementary Deoxyribonucleic Acid

CEB1: Cyclin E-Binding Protein 1

cGMP: Cyclic Guanosine Monophosphate

CHX: Cycloheximide

DAPI: 4',6-Diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic Acid

E1: Ubiquitin Activating Enzyme

E2: Ubiquitin Conjugating Enzyme

E3: Ubiquitin Ligase

E4: Ubiquitin Elongation Enzyme

E6AP: E6- Associated Protein

ECL: Enhanced Chemiluminescence

EDTA: Ethylenediamine Tetraacetic Acid

EGFP: Enhanced Green Fluorescent Protein

EGTA: Ethylene Glycol Tetraacetic Acid

eIF: Eukaryotic Translation Initiation Factor

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GAS: Gamma Activated Sequence

GDP: Guanosine Diphosphate

GEF: Guanine Exchange Factor

GFP: Green Fluorescent Protein

HECT: Homologous to E6-AP Carboxy Terminus

HEK: Human Embryonic Kidney

HERC: HECT and RCC1 Containing

HHV: Human Herpes Virus

HIV: Human Immunodeficiency Virus

HSV: Herpes Simplex Virus

hTERT: Human Telomere Elongation Reverse Transcriptase

IAP: Inhibitor of Apoptosis

ICP0: Infected Cell Protein 0

IFITM1: Interferon-Inducible Transmembrane Protein 1

IFN: Interferon

IFNaR: Interferon Alpha Receptor

IFNgR: Interferon Gamma Receptor

IgG: Immunoglobulin Gamma

IkBa: Inhibitor of κB Alpha

IL1b: Interleukin 1 Beta

IRF: Interferon Response Factor

ISG: Interferon Stimulated Gene

ISGF3: Interferon Stimulated Gene Factor 3

ISRE: Interferon Stimulated Response Element

IU: International Units

JAK: Janus Kinase

kBP: Thousand of Base Pairs

LPS: Lipopolysaccharide

MHC: Major Histocompatibility Complex

mRNA: Messenger Ribonucleic Acid

NCBI: National Center for Biotechnology Information

NFkB: Nuclear Factor κB

NK: Natural Killer

OAS: Oligoadenylate Synthetase

PAM: Protein Associated with c-Myc

PAMP: Pathogen Associated Molecular Pattern

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PenStrep: Penicillin and Streptomycin

PFU: Plaque Forming Units

pGQ: Plasmid Engineered by Graeme Quest

PLK: Polo-Like Kinase

POI: Protein-of-Interest

Poly I:C: Poly-Inosinic: Poly-Cytidylic Double-Stranded Ribonucleic Acid

pRB: Protein Retinoblastoma

qRT PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction

RCC1: Regulator of Chromosome Condensation 1

RING: Really Interesting New Gene

RJS: Runty, Jerky, Sterile Syndrome

RLD: Regulator of Chromosome Condensation 1-Like Domain

RNA: Ribonucleic Acid

RNase: Ribonuclease

rRNA: Ribosomal Ribonucleic Acid

RT PCR: Reverse Transcriptase Polymerase Chain Reaction

SAGE: Serial Analysis of Gene Expression

SDS: Sodium Dodecyl Sulphate

SDS-PAGE: Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis

STAT: Signal Transducer and Activator of Transcription

SUMO: Small Ubiquitin-Like Modifier

SV40; Simian Virus 40

TAE: Tris Acetate EDTA Buffer

TE: Tris EDTA Buffer

TK: Thymidine Kinase

TLR: Toll-Like Receptor

TNFa: Tumour Necrosis Factor Alpha

TRE: Tetracycline Response Element

tRNA: Transfer Ribonucelic Acid

TTBS: Tris-Buffered Saline with Tween 20

TUNEL: Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick End-Labelling

UAE: Ubiquitin Activating Enzyme

UBC: Ubiquitin Conjugating Enzyme

UbcH: Human Ubiquitin Conjugating Enzyme

UBL: Ubiquitin Like Protein

UCRP: Ubiquitin Cross-Reactive Protein

UEE: Ubiquitin Elongation Enzyme

UEV: Ubiquitin Conjugating Enzyme E2 Variant

VSV: Vesicular Stomatitis Virus

Chapter 1: Introduction

1.1 Intracellular Antiviral Responses

1.1.1 Initiation of an Antiviral Response

Inherent to the survival of organisms, cells must be able to respond to changes within and without in order to persist. Organisms must prevent drastic changes in intracellular pH or osmolarity in order to maintain function; similarly, cells must also be able to react to and suppress viral infection. Just as sensory mechanisms are in place to detect intracellular and extracellular changes in pH and osmolarity, mammalian cells encode mechanisms to detect viruses (and other pathogens) and initiate a response. Through evolutionary adaptation, mammalian cells bear numerous receptors: such as Toll-like receptors (TLRs) [7, 33, 257, 292], NOD-family proteins [7, 158, 159, 257], double-stranded RNA–activated kinase (PKR) [257, 419, 420], and DExH RNA helicases (such as RIG-1 and MDA-5) [53, 257, 429, 430], to detect pathogen-associated molecular patterns (PAMPs). Many PAMP-receptor interactions have been described, including the detection of viral glycoproteins and capsid components by Toll-like receptors (TLRs) 2 and 4 [23, 28, 44, 65, 87, 91, 137, 202, 203, 392, 412], viral genomic nucleic acids by TLRs 3, 7, 8, and 9 [3, 85, 128, 139, 144, 195, 230, 231, 358, 382, 393, 413], as well as double-stranded RNA by RIG-1, TLR3, and PKR [3, 11, 37, 128, 177, 197, 199, 358, 382, 413, 426, 429, 430]. Additionally, infection of human cells by enveloped viruses appears to induce an antiviral response, though the mechanism(s) of its initiation is yet to be identified [64].. The stimulation of such receptors can potentiate signal transduction and subsequent activation of latent transcription factors in the cytoplasm, such as NFκB, ATF-2, c-Jun, and IRF-3, resulting in their translocation to the nucleus and transactivation of genes encoding other transcription factors, cytokines, and various effector proteins involved in bolstering and propagating the antiviral response (Figure 1.1). As these transcription factors are latently present in the cytoplasm of cells, their activation and subsequent activation is independent of translation, thus genes activated by these factors may be termed primary antiviral response genes.

1.1.2 Autocrine and Paracrine Signalling in Antiviral Responses

During an intracellular antiviral response, numerous signalling molecules are transcribed, translated, and secreted into the extracellular milieu. The release of such cytokines and chemokines has three roles: to amplify the antiviral response of the infected cell, to pre-emptively induce antiviral states in neighbouring cells, and to recruit leukocytes to the area and activate an immune response. The first cytokines to be described [42, 160, 161, 223], the interferons, bind to their cognate receptors to initiate the transcriptional activation of numerous antiviral gene products. This transactivation is mediated through the activation of latent cytoplasmic transcription factors, namely members of the STAT (signal transducers and activators of transcription) family [375].

Interferons are sub-divided into two families: type I (innate) and type II (immune) interferons. The type I interferon family, in humans, consists of five members: interferons α, β, ε, κ, and ω, all of which are encoded in a locus at chromosome 9q21-22 [41, 256]. Humans encode only a single gene for each of interferons β, ε, κ, and ω, while 13 functional genes (and one pseudogene) encode subtypes of interferon α [134]. While all type I interferons bind the type I interferon receptor, it is of note that members of the type I interferon family (and for interferon α, even amongst subtypes) have differential abilities to activate antiviral responses [295]. All cells capable of transcription are thought to be able to produce type I interferons, albeit preferences for subtypes differ by cell type [24, 151, 308, 390].

The type I interferon receptor, which recognizes all type I interferons, is minimally composed of two transmembrane proteins, IFNαR1 and IFNαR2c. The cytoplasmic domains of the type I interferon receptor complex recruit STAT1 and STAT2, in addition to two members of the Janus kinase (JAK) family of tyrosine kinases, Jak1 and Tyk2 [41, 256]. Upon ligand binding and dimerization of the type I interferon receptor, the JAK family kinases are activated, leading to the tyrosine phosphorylation of STAT1 and STAT2, facilitating their homo- or heterodimerization. Upon dimerization, STATs translocate to the nucleus to be active as transcription factors. STAT1 homodimers are able to bind to gamma-activated sequence (GAS) elements, and in cooperation with the transcriptional coactivator CBP/p300, can activate a subset of interferon-stimulated genes (ISGs) including the transcription factor interferon response factor (IRF) 1. STAT1, STAT2, and IRF9 (also called p48) heterotrimerize to form interferon-stimulated gene factor 3 (ISGF3). Binding of ISGF3 to interferon-stimulated response elements (ISREs), in cooperation with CBP/p300, leads to the transcriptional activation of numerous ISGs and initiation of an antiviral response.

Only one type II interferon exists in humans, interferon γ, which is encoded at chromosome 12q14. Unlike type 1 interferons, which are active as monomers, interferon γ is active as a tetramer [294]. Primarily produced by T and NK lymphocytes, interferon gamma specifically binds the type II interferon receptor, composed of the transmembrane proteins IFNγR1 and IFNγR2. The cytoplasmic domains of these proteins recruit STAT1 homodimers [36], as well as two members of the Janus kinase (JAK) family of tyrosine kinases, Jak1 and Jak2. Binding of interferon γ induces the dimerization of the type II interferon receptor, consequently inducing the activation of JAK family kinases. Active Jak1 and Jak2 phosphorylate the STAT1 homodimers on multiple tyrosine residues, resulting in the translocation of STAT1 homodimers (termed gamma-activated factor) to the nucleus to be active as a transcription factor. STAT1 homodimers are able to bind to gamma-activated sequence (GAS) elements, and in cooperation with the transcriptional coactivator CBP/p300, can activate a subset of interferon-stimulated genes (ISGs) including the transcription factor interferon response factor (IRF) 1.

As the required transcription factors (STATs) are latently present in cells, no translation is required for the transcriptional activation of genes through the activation of these factors. Thus, genes activated by these factors (through GAS elements or ISREs) may be referred to as primary ISGs.

1.1.3 The Antiviral Transcriptional Cascade

The activation of an antiviral response occurs in phases of gene activation. Through the primary activation of PAMP-recognition molecules, transcription factors such as IRF-3, NFκB, c-JUN, and ATF-2 may activate a subset of interferon-stimulated genes (dependent on IRF-3 binding the ISRE of responsive genes), including interferon β (Section 1.1.1).

Production of interferon β can activate the type I interferon receptor intracellularly [363], and thereby stimulate the activation of STATs. Secretion of interferon β into the intercellular milieu facilitates the interaction of the cytokine with the type I interferon receptor on the producing cell (autocrine positive feed-back) or on neighbouring cells leading to the paracrine activation of STATs and transcription of interferon-responsive genes. Binding of ISGF3 to ISREs in target genes may function to further or continue activation of genes stimulated during the primary activation of antiviral genes, but also functions to activate several genes unique from that response.

One of the genes transcribed in response to ISGF3-dependent gene activation, IRF-7, is a potent transcription factor. In conjunction with ISGF or IRF-3, IRF-7 allows for the activation of further ISGs, including the interferon α genes. Interferon α can activate the type I interferon receptor either intracellularly [1, 334], on the secreting cell (autocrine positive feed-back), or on local cells to stimulate the activation of STATs in these cells. As a direct consequence through the activation of STATs, the production of IRF-7 in interferon-stimulated cells can result in interferon α production in these cells – thereby propagating the antiviral response. As the transcription of the interferon α genes, and other ISGs requiring IRF-7 for activation, is erstwhile dependent on the *de novo* translation and activation of IRF-7, these genes may be referred to as secondary ISGs.

1.1.4 Antiviral Effector Proteins

The identification and elucidation of function of antiviral proteins is ongoing. While numerous proteins are now recognized to be regulated as part of the antiviral response, the function of many of these proteins remains unknown. Of the characterized members of the family of antiviral effector proteins, several themes of function are apparent: inhibition of protein synthesis, enhanced proteolysis and increased antigenic presentation, and inhibition of proliferation.

Two antiviral effectors functioning to inhibit translation are currently known. Interaction of PKR with double-stranded RNA or the cellular protein PACT [293] triggers activation of PKR through its dimerization (homodimerization or heterodimerization with PACT) and subsequent autophosphorylation [83]. Following activation, PKR interacts with numerous cellular proteins, such as IRAK and TAK1, thereby activating downstream signalling pathways (Figure 1.1.1). In addition to its signalling functions, PKR acts as an effector by interacting with and phosphorylating eukaryotic initiation factor 2α (eIF2α) [83, 84]. Phosphorylation of eIF2α effectively stabilizes its interaction with GDP and prevents the activity of eIF2β, a guanine exchange factor (GEF). In the GDP-bound form, eIF2 is unable to recruit methionyl tRNA, and therefore unable to catalyze the initiation of translation. A further blockade to translation is effected by ISG 56k, which binds the p48 subunit of eIF3 [129, 154]. Should the eIF2 complex escape phosphorylation by PKR and successfully recruit methionyl tRNA, ISG 56k effectively blocks the recruitment of eIF3 to this complex and thereby halts further progression of translation initiation.

Upstream of translation, components of the antiviral response increase degradation of mRNA. Similar to PKR, the 2', 5' oligoadenylate synthetase (OAS) family of proteins are activated upon binding to double-stranded RNA, and subsequent to their activation catalyze the formation of 2', 5'-linked oligoadenylate chains [178, 179]. While multiple, distinct OAS proteins are encoded and activated during the antiviral response [77, 210, 259], the differential roles of the isozymes are not yet elucidated. Binding of the 2', 5' oligoadenylates to RNase L induces its dimerization and activation, consequently cleaving rRNAs and mRNAs [9, 61, 104, 265, 313]. By targeting both rRNA and mRNA, activation of RNase L functions to inhibit ribosome function and to decrease the abundance of message for translation.

As viruses require host translational machinery, these blockades function to quickly cease the production of viral proteins. In order to deal with viral proteins produced which escape this blockade, cells increase protein turnover. One mechanism to achieve this is through the upregulation of components of the ubiquitin-proteasomal pathway [210, 275] - a cellular pathway which leads to the selective targeting of proteins for proteolysis. Increasing the rate of proteolysis (via increased abundance and activity of ubiquitin pathway components) can enhance antigen presentation by class I MHC [126, 251]. Class I MHC is present on nearly all nucleated cells [172], and functions to present antigens from within the cell to patrolling lymphocytes. Antigen recognition in the context of class I MHC may potentiate activation of adaptive ($CD8^+$ T cells) lymphocytes, while a lack of class I MHC presentation (which viral proteins may inhibit) may activate innate lymphocytes (Natural Killer cells). Components of the class I MHC-antigen presentation pathway are also upregulated during an antiviral response [210, 252, 259].

As both cellular replication and antiviral responses require large amounts of energy, initiation of an antiviral response is accompanied by inhibition of cellular proliferation [13]. To these ends, interferons have been shown to down-regulate or inhibit numerous proteins associated with cell cycle progression or bolster those with cell cycle inhibitory function [200, 338-340, 363, 378, 388]. Additionally, pro-apoptotic pathways may be sensitized or activated as a part of the antiviral response, leading to the degradation of nucleic acids and proteins, loss of mitochondrial potential, and death of the cell [12, 20, 55, 125, 338].

1.2 The Mammalian Cell Cycle

1.2.1 Cyclins and Cyclin-Dependent Kinases

The mammalian cell cycle is driven by the coordinated and temporal activation of cyclins-dependent kinases (CDKs), and held in check by cell cycle checkpoint pathways and inhibitors of cyclin-dependent kinases. Cyclin-dependent kinases represent a family of serine/threonine kinases, which by themselves are inactive. The specificity and regulation of kinase function of

CDKs is provided through heterodimerization with cognate cyclins. Cyclins A, B, C, D, and E are temporally regulated through the cell cycle, with the transcription, activation, and activity of one cyclin-CDK holoenzyme contributing to the transcriptional activation of the next temporal class, thereby pushing the cell cycle. It is of importance to note, however, that studies of knockout mice lacking single cyclins or cyclin-dependent kinases suggest that loss of single functions are either compensated for by other cyclins, cyclin-dependent kinases, or kinases, or are non-essential [21, 35, 60, 103, 116, 192, 226, 239, 243, 263, 285, 289, 311, 365, 366, 396]. Cell cycle progression is diagrammed with the cyclin-CDK holoenzyme(s) dominant in that phase (Figure 1.2.1).

Cyclin-CDK holoenzymes are regulated by multiple mechanisms, as befitting the importance and consequence of their function. Just as transcriptional regulation of cyclins give rise to the cyclical activation patterns of CDK holoenzyme activity, the cessation of activity can be achieved by the degradation of the cyclin. To these ends, the conjugation of poly-ubiquitin chains to cyclins (as well as many other cell cycle regulators) by the temporal activation of ubiquitination pathways leads to the targeted degradation of such proteins by the proteasome.

In addition to transcriptional and proteolytic regulation, CDK holoenzymes are also regulated by reversible processes. Following formation of the holoenzyme, full activation of kinase function is mediated by threonine phosphorylation at a conserved residue of the "T loop" (T161 in CDK1, T160 in CDK2), which obstructs the CDK active site [73, 133, 206, 248]. This phosphorylation is thought to be mediated by a complex termed the CDK-activating kinase (CAK), composed of CDK7, cyclin H, and ménage-a-trois 1 (MAT1) [81, 106, 206]. Dephosphorylation of the conserved threonine residue appears to be mediated by kinase-associated phosphatase 1 (KAP1) following cyclin dissociation [304].

In contrast, phosphorylation of CDKs on amino-terminal threonine and tyrosine residues (T14 and Y15 in both CDK1 and CDK2), which reside in the ATP-binding loop, acts to inhibit kinase activity. Phosphorylation of these residues are mediated by Myt1 (both T14 and Y15, CDK1 specific [29]) and Wee1 (Y15 only) [227, 249, 417]. A family of dual-specificity phosphatases, cdc25 proteins, function to dephosphorylate T14 and Y15 modifications, thereby activating the CDK holoenzyme. Three cdc25 family members, named cdc25A, cdc25B, and cdc25C, are recognized in mammalian cells, each of which bears a unique localization, temporal transcription pattern, and mechanisms for regulation of its activity [88].

1.2.2 Cell Cycle Checkpoints and Regulation

In order to ensure that all required processes are complete prior to cell cycle progression, cell cycle checkpoints are required to guard against premature entry at every cell cycle phase. Cell cycle progression from G0 through G1 phase is initiated by mitogenic stimulation, leading to the transcription of cyclin C and D-type cyclins [215, 314, 359]. However, as commitment to cellular replication is irreversible, checkpoints need to ensure there is sufficient mitogenic stimulation to proceed. The retinoblastoma tumour suppressor protein (pRB) is one such checkpoint control. Retinoblastoma protein binds to E2F-family transcription factors and consequently inhibits their ability to transactivate responsive genes. Current theories propose that cyclin C-CDK3, followed by D-type cyclins in conjunction with CDK4 and CDK6, act to effect (though perhaps indirectly [317]) successive phosphorylations on pRB [314, 360]. During the peak of D-type cyclin-CDK activity, cyclin E is induced, and in activating CDK2 leads to the hyperphosphorylation of pRB (and other members of the "pocket protein" family) and the release of E2F transcription factors. Numerous genes products are induced by E2F transcription factors, including many proteins associated with DNA replication, transcription factors such as c-myc, and cyclin E. The enhanced production of cyclin E represents an amplification loop for cyclinE-CDK2 activity, and acts to commit the cell to progression from G1 to S phase, and onward to eventual mitosis.

Though the cell is committed to mitosis following entry into S-phase, it is sometimes essential that cell cycle progression be paused. As maintenance and error-free replication of cellular DNA are critical to the health of cells, all errors and insults must be dealt with prior to continuing towards cell division. Examples of such situations are damage to the cellular DNA by ionizing radiation, ultraviolet radiation, chemical DNA damaging agents, or incomplete replication during S phase. Due to the extreme importance of the genetic material, numerous pathways are present to detect such insults and errors, though two responses are currently recognized: p53-dependent and independent mechanisms.

Downstream of the detection of DNA damage, the tumour suppressor p53 is activated by phosphorylation by one or more upstream kinases (such as ATM in response to ionizing radiation, and ATR in response to ultraviolet radiation). Activating phosphorylation of p53 promotes enzymatic conformational changes [433, 442], facilitating tetramerization and DNA-binding ability [163, 301, 408, 433, 442], allowing it to act as a potent transcription factor. Activated p53 stimulates numerous genes involved in DNA repair, as well as numerous other genes involved in arresting the cell cycle. Of these genes, $p21^{CIP1}$ (also called cdkn1a or Waf1) is perhaps the best characterized. Characteristic of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs), $p21^{CIP1}$ bears an amino-terminal CDK inhibitory domain which is broadly specific for (at least) CDKs 1, 2, 4, and 6. Expression of $p21^{CIP1}$ at high levels is sufficient to block CDK activity, though lower levels may stimulate CDK-cyclin association [205]. Additionally, $p21^{CIP1}$ has been shown to bind proliferating cell nuclear antigen (PCNA), a component of DNA polymerases δ and ε, and efficiently block PCNA-dependent DNA replication but not repair function [220]. Evidence also links cytoplasmic-localized CIP/KIP family members to regulation of the cytoskeleton [76].

As an additional safety mechanism, activation of chk1 and chk2 by DNA-damage sensing kinases (such as ATM and ATR) leads to the phosphorylation of the CDK-activating cdc25 proteins, thereby inhibiting CDK activation [99, 228, 235, 372, 440, 441]. Following phosphorylation of cdc25 proteins, they become destabilized and either targeted for ubiquitination and proteasomal degradation, or alternatively, chelation by 14-3-3 proteins and inhibition of function [88].

Beyond DNA damage, cell cycle progression may also be arrested in response to spindle microtubule damage. In order for chromosomes to be correctly aligned and segregated during mitosis, the spindle microtubules must be properly formed (*i.e.*, complete and polarized) with the kinetochores anchoring sister chromatids to opposite poles. Incorrectly polarized or incomplete spindles, as well as chromatids unattached to spindles, lead to catastrophic errors as mitosis continues. To prevent such occurrences, kinetochores recruit spindle checkpoint kinases hMps1 (also termed TTK kinase), BubR1, and Bub1, as well as Mad1 and Mad2 proteins [190]. BubR1 is recruited to unattached kinetochores via centromere protein E (CENP-E), which when unbound to a spindle microtubule induces the activation of BubR1 [241]. Activated BubR1 facilitates the recruitment of Mad1:Mad2 heterodimers, which then recruit monomeric Mad2 and induce a conformational change in it [241]. This activated conformation of Mad2 facilitates interaction with cdc20, an essential activator of the anaphase-promoting complex/cyclosome (APC/C), thereby inhibiting the initiation of anaphase [100, 232]. Similarly, activated BubR1 is also able to chelate cdc20 from the APC/C to inhibit initiation of anaphase [51]. Unattached kinetochores also recruit hMps1, a multi-specific serine/threonine/tyrosine kinase, which plays a critical role in the recruitment of Mad1 to kinetochores [229, 245] and is also known to interact with the APC/C [229].

As sister chromatids associate with anchored spindle microtubules, microtubule motors induce tension in the microtubules while cohesins act to hold the sister chromatids together. This tension and kinetochores-microtubule occupancy acts to silence spindle checkpoints at that junction. As kinetochores and spindles associate, aurora kinases act to induce cleavage of spindle microtubules which fail to generate appropriate tension with kinetochores, thereby recreating unattached kinetochores. [4]. While the role of polo-like kinases (PLKs, which are recognized to regulate cdc25C [288, 325, 391]) is not yet clearly defined in the regulation of spindle checkpoints, they appear to play a critical role in the proper formation and maintenance of mitotic spindles and progression through mitosis [208, 262, 288, 325, 353, 391].

When all kinetochores-spindle interactions have produced sufficient tension and thereby inactivated all spindle checkpoints (*i.e.*, completed metaphase), cdc20 may associate and activate the APC/C [241]. Activation of the APC/C by binding cdc20 and phosphorylation by cyclin B-CDK1 stimulates its function as a ubiquitin ligase [297]. Subsequent to its activation, the APC/C promotes the ubiquitination and subsequent proteasomal degradation of cyclin B and securin. Securin acts to inhibit the protease separase, which functions to cleave a cohesin (hRad21) holding sister chromatids together [148], thereby initiating anaphase. Following loss of cyclin B, the APC/C may alternatively associate with cdh1, which functions to target cdc20, as well as cyclins A, B, and E for degradation [117, 298]. These events ensure exit from mitosis and dependence on mitogen-stimulated cyclin expression for cell cycle re-entry.

1.2.3 A Requirement of Cell Cycle Factors for Viral Replication

As obligate parasites, the replication of viruses is intimately tied to many cellular processes and enzymes. As such, it comes as little surprise that many viruses have adapted to utilize cell cycle factors. Indeed, members of the smaller DNA virus families (parvoviridae, papillomaviridae, and polyomaviridae) are dependent on the production of host S phase proteins including host DNA replication machinery [186]. Parvoviruses have an unusually passive mechanism to obtain access to cellular replication machinery: they simply wait until the host cell enters S phase and then hijack the needed proteins for their own purposes [186]. Thus, without host cell cycle progression or compensation by host co-infection with a helper virus (adenoviruses or herpesviruses), parvoviruses are incapable of replication.

Unlike parvoviruses, many other nuclear DNA viruses take a more active role in initiating their replication. As described in section 1.2.2, the activation of E2F transcription factors is one of the key events which commits a cell to progression into S phase and concomitantly leads to the production of cellular DNA replication machinery. Members of adenoviridae, papillomaviridae and polyomaviridae utilize similar mechanisms to achieve such ends. In order to activate E2F transcription factors, each of these virus families encodes a protein (E1A, E7, and large T antigen, respectively) which chelates the E2F-binding pocket of pocket proteins (pRB, p107, p130), thereby freeing E2F factors for transcriptional activation [56].

These viral families additionally block the activity of p53, effectively preventing the initiation of a cell cycle block and inhibition of replication machinery. Towards this, each viral family uses a different mechanism with largely the same ends. Adenoviruses encode the E1B 55k protein, which binds to p53 but does not prevent its DNA binding ability. Rather, E1B 55k effectively block the transactivation domain of p53, thereby converting p53 into a transcriptional repressor [244, 341]. Papillomaviruses encode the E6 protein, which recruits a cellular E3 ubiquitin ligase (E6-associated protein (E6-AP)), which in conjunction lead to the poly-ubiquitination and subsequent proteolytic degradation of p53 [346, 418]. Polyomaviruses utilize large T antigen to bind p53 and block its ability to bind DNA (and therefore, to act as a transcription factor) [14, 207, 225]. Thus, while the mechanism is unique to each viral family, members of adenoviridae, papillomaviridae, and polyomaviridae effectively ablate the ability of p53 to activate target genes.

Evidence suggests that these viruses also block the activity of CIP/KIP family cyclin-dependent kinase inhibitors [2, 108, 173, 236, 305, 435], which suggests that CDK function may be required by these viruses in addition to activation of E2F transcription factors. Recent publications have demonstrated that CDKs play essential roles in replication of each of these virus families [45, 70, 124, 217, 221, 234, 254, 394]. The application of pharmacological cyclin-dependent kinase inhibitors to study the potential requirement of CDKs for viral replication has yielded interesting results: in addition to adenoviridae, papillomaviridae, and polyomaviridae, herpesviridae and human immunodeficiency virus (HIV) have been shown to require CDK activity for their replication (reviewed in [343]). As both alpha herpesviruses and HIV are able to replicate in non-dividing cells it is not immediately apparent why CDK activity is required for replication, nor for transcription of viral genes [38, 39, 196, 343-345, 386, 410]. While not yet fully elucidated, it appears that CDKs and cell cycle regulatory factors are intimately involved in the lifecycle of all nuclear DNA viruses and perhaps those of other viral families as well [343].

1.2.4 Modes of Cell Death

Just as humans and other metazoans age, acquire injuries, and suffer infections, so do individual cells. As the unregulated growth of a cell in a metazoan organism may result in malignant disease, interference of organ function and death of the organism, the death of such cell is of benefit to the organism. Similarly, cells infected by pathogens allow for the replication and spread of such parasites throughout the host organism, placing the fate of all cells in jeopardy. Again, the death of the infected cell may save the host from disease and death. Due to the grave importance of the overall health of the organism, numerous mechanisms are present to terminate cells which may be dangerous or unnecessary, a few of which will be described here.

1.2.4.1 Apoptosis

Apoptosis is a cellular process during which cells initiate a cascade of events culminating in the non-inflammatory death of the cell. Apoptosis is a complex and highly regulated process characterized by numerous biochemical and morphological phenomena. The apoptotic phenotype is characterized by the loss of mitochondrial membrane potential, condensation of chromatin, degradation of chromatin to nucleosome-sized fragments, activation of cellular proteases, and ruffling or blebbing of the plasma membrane [98, 180, 193, 423, 434]. Apoptosis may be initiated from without, such as by the engagement of cellular “death receptors” (*e.g.*, by interaction of the cellular Fas receptor (also referred to as APO-1 or CD95) with Fas ligand (also termed CD95L)) as potentiated by activation of immune cells [102, 329, 371, 384, 425]. Alternatively, apoptosis may be initiated from within in response to grave cellular insults, such as DNA damage, infection, or growth factor withdrawal.

When initiated by intrinsic signalling events, the commitment to apoptosis hinges on the integrity of the mitochondria. Following suitable stimulation, BH3 domain-only proteins (such as BID, BAD, BIM, and PUMA) are activated by mechanisms peculiar to each [78, 266, 276, 306, 307, 415, 432, 436]. Activation of such BH3 domain-only proteins enables their interaction with other members of the BCL-2 family. BCL-2 family proteins are characterized by their homology to domains of the BCL-2 protein, named BCL-2 homology domains one through four (BH1-4). Pro-apoptotic members of the BCL-2 family contain BH1, 2, and 3 domains, and include BAK and BAX which appear to be essential for the intrinsic activation of apoptosis [224, 415]. Activated BH3 domain-only members lead to the activation of pro-apoptotic members of the BCL-2 family [214]. Activation of BAK or BAX stimulates conformational changes leading to their homo-oligomerization. Though the mechanism remains incompletely elucidated, homo-oligomerization of BAK and/or BAX leads to the permeablization of the mitochondrial outer membrane with the subsequent release of proteins from the inter-membrane space [5, 185, 204, 287, 350, 414]. In contrast, anti-apoptotic members of the BCL-2 family bear all four BH domains, and include BCL-2, BCL-xL, BCL-W, MCL-1, and A1/BFL-1. Anti-apoptotic BCL-2 family members function, at least in part, to inhibit the effects of BH3 domain-only and pro-apoptotic BCL-2 family members by binding and sequestering these proteins, thereby inhibiting the homo-oligomerization of BAK or BAX and subsequent permeablization of the outer mitochondrial membrane [57, 127, 146, 183, 332, 444]. As such, activation of apoptosis by intrinsic stimuli occurs when the activation of BH3 domain-only family members outweighs the abilities of anti-apoptotic BCL-2 family members to suppress the activation of pro-apoptotic BCL-2 family members.

Upon permeablization of the mitochondrial outer membrane, cytochrome C is released into the cytoplasm, where it binds to and induces the oligomerization of apoptotic protease activating factor-1 (APAF-1) to form the apoptosome [219, 445]. With ATP, the apoptosome catalyzes the activation of caspase 9, a member of a family of cysteine proteases which are activated by site-specific proteolytic cleavage [270]. Caspase 9 acts as an initiator caspase, activating downstream effector caspases (such as caspases 3, 6, and 7) by proteolytic modification. Activation of effector caspases consequently results in the proteolysis of numerous target proteins. While the significance of many targets remains obscure, several others have been directly linked to the progression of apoptosis. Cleavage of PAK2/hPAK65, a member of the p21 Rho-activated kinase family (which interact with cytoskeletal GTPases), leads to the constitutive activation of this kinase [211, 331]. While its targets are not yet known, over-expression of PAK2/hPAK65 kinase domain lead to many of the hallmarks of apoptosis, such as nuclear condensation, cellular detachment, and phosphatidylserine externalization on the plasma membrane [211, 331]. Nuclear lamins, fundamental structural elements of the nuclear membrane, are targets of caspase 6 [286, 312, 383]. Expression of lamins lacking caspase cleavage sites prevents or delays chromatin condensation, DNA fragmentation, and nuclear fragmentation [312]. Caspases also lead to the activation of caspase-activated deoxyribonuclease (CAD) by proteolytic inactivation of its repressor (ICAD/DFF-45), subsequently allowing the nuclear import of CAD and subsequent degradation of DNA [94, 335]. Thus, caspases regulate processes which result in the apoptotic phenotype.

Apoptosis is further regulated through the inhibition of caspases. Caspase activation and activity is controlled by cellular (or viral) inhibitors of apoptosis (IAPs), such as XIAP, c-IAP1 and c-IAP2 [82, 330], as well as BCL-2 and BCL-xL [216, 326, 443]. In turn, IAPs are also post-translationally regulated by IAP-inhibitory proteins such as SMAC/DIABLO and OMI/HTRA2 [90, 138, 246, 380, 403, 404]. Clearly, the purposeful and controlled progression towards cell death by apoptosis is energy intensive and highly complex, but ensures inactivation of viruses through the degradation of nucleic acids and activation of proteolytic enzymes.

1.2.4.2 Mitotic Catastrophe

Sustained checkpoint failures during mitosis may trigger a form of cell death which may include many of the biochemical hallmarks of apoptosis, and also others that mark it as a distinct form of cell death [47, 48, 281, 322]. Following damage to the cytoskeleton or cellular DNA, or failure to inactivate cellular replication checkpoints, cells initially arrest cell cycle progression in an attempt to repair damage or errors. Should the cellular insult be sufficient, apoptotic programs may be initiated through p53-dependent or independent pathways. However, cell cycle progression may continue to mitosis in cells which bear inactive checkpoints and consequently fail to initiate apoptosis or sustain cell cycle arrest, despite grave damage,. This progression results in aberrant segregation of chromosomes during anaphase, frequently resulting in the formation of micronuclei. Indeed, nuclear fragmentation and the formation of micronuclei, along with distortions in nuclear structure, are some of the morphologic features associated with mitotic catastrophe [47, 281, 322]. As a consensus on the formal definition has not yet been reached, it remains vague what biochemical features constitute mitotic catastrophe [193]. The involvement of caspases and later activation of apoptosis versus caspase-independent cell death are contested [193]. Despite the vague understanding of the mechanism of mitotic catastrophe, numerous studies have implicated dysregulation of cell cycle and cytoskeletal proteins in the progression and morphology of mitotic catastrophe [43, 49, 52, 58, 62, 96, 107, 152, 240, 271, 274, 353, 354, 356, 368, 381, 395, 401, 407, 421, 437, 447]. The notable proteins associated with mitotic catastrophe, including under-regulated cyclin B: CDK1 activity, chk1 and chk2 checkpoint deficiency, lack of p53 effectors (such as $p21^{CIP1}$ or 14-3-3σ), and dysregulation of microtubule regulation factors, illustrate that cell cycle checkpoints and mitotic progression events are critical to this type of cell death.

1.2.4.3 Necrosis

Necrosis is a mode of cell death which may result from a loss of cellular bioenergetics, and as such may represent a default mechanism [93, 136, 213].

Many stimuli which are capable of inducing apoptosis may also result in necrotic death [361, 362]. Morphologically characterized by cytoplasmic swelling, permeablization of the plasma membrane, and dilation of cellular organelles, necrotic death results in the loss of cellular maintenance functions, organelle function, and membrane integrity [193]. As opposed to apoptosis, whereby cellular contents are degraded in a controlled manner and remain contained within the apoptotic body, necrosis represents a deregulated form of death where cellular decomposition results in the eventual bursting of the cell and release of contents into the extracellular milieu [351]. The release of cellular contents results in local inflammation, potentially initiated by the recognition of intracellular proteins by extracellular Toll-like receptors [6, 218, 277, 387, 399, 400]. While inflammation may cause further damage to neighbouring cells, it also functions to recruit and activate phagocytes and other immune cells. Should foreign proteins or other markers of infection be present in the context of necrosis, their uptake and antigenic presentation to immune cells may function to further both innate and adaptive immune responses [6, 15, 22, 109, 218, 277, 342, 387, 399, 400].

1.3 HERC Family Proteins

1.3.1 The HERC Protein Family

The HERC protein family is characterized by the presence of one or more RCC1-like domains (RLDs) at the amino terminus of the protein, coupled with a homologous to E6-associated protein carboxy terminus (HECT) domain. Thus far, six members of the HERC protein family have been described, and have been separated into the subfamilies of large HERC and small HERC proteins [143]. Two large HERC proteins have been described thus far, HERC1 (also titled p619 and p532) and HERC2, both approximately 500 kDa in size. These proteins contain two and three RLDs in their amino termini, respectively, and contain additional protein domains (distinct to each protein) between the RLDs and HECT domain. The four small HERC proteins, HERC3 (also called KIAA0032 or D25215), HERC4 (also termed KIAA1593 or AB046813), HERC5 (previously referred to as cyclin E-binding protein 1, CEB1, or confusingly, HERC4), and HERC6, are each approximately 120 kDa in size. Each bears a single RLD in its amino terminus, and contains no other recognized protein domains save for the HECT domain at the carboxy terminus.

Based on genomic information, HERC family proteins appear unique to animalia, as they are not encoded in plant or fungi genomes, though proteins containing RLDs or HECT domains are [113]. Based on phylogenetic data, it is speculated that the HERC protein family arose from a single ancestral gene, most closely related to HERC4 (which has a *Caenorhabditis elegans* orthologue, stemming out from the basal line of the HERC phylogenetic tree) [113, 143]. HERC2 is present in chordates, as well as arthropods, but not nematodes. This suggests that HERC2 may have been encoded by in a common ancestor of protostomes and deuterostomes, but the gene was lost in nematodes [113], or alternatively, that HERC2 arose from convergent evolution in arthropods and chordates, but not nematodes [143]. HERC1 and HERC3 appear to have arisen with teleosts, while HERC6 seems to first appear in rodents [143]. It is interesting that the HERC proteins are highly conserved between humans and mice, bearing greater than 90% identity, with the exceptions of HERC6 (66% identity) and HERC5 [113, 143]. HERC5 is a gene apparently unique to primates [143].

While the phylogenetic data supports the separation of the HERC proteins into large and small subfamilies, it is not readily clear whether the small and large HERC proteins arose separately or whether one of the subfamilies arose through gene duplication and recombination events from the other [113, 143]. Nevertheless, genomic and phylogenetic data supports HERC gene duplication and divergence as an ongoing process in animalia, with HERC4 appearing to give rise to HERC3, and HERC3 giving rise to HERC6 [113, 143]. HERC5 appears to have recently arisen from HERC6, as these genes bear a greater similarity to each other than to each other than to HERCs 3 or 4 [113, 143]. It is interesting that HERCs 3 and 6 (and HERC5, where present) occur in the same syntenic region (in humans, chromosome 4q21-22) in all species analyzed thus far [113, 143]. HERC4 maps alone in all species (in humans, to chromosome

10q22), while HERCs 1 and 2 are present on the same chromosome, but map to different synteny regions (in humans, to chromosome 15q22 and 15q13, respectively) [113, 143]. Evidence of ongoing gene duplication and recombination has been reported for HERC2, with several partially-duplicated paralogues of HERC2 found on human chromosomes 15 and 16 [168].

While little is known about the functions of HERC family proteins (especially for HERC4 and HERC6, the most recently identified members) roles in vesicle trafficking and the cytoskeletal modulation have been suggested. While the localization of HERC2 has yet to be described, all other HERCs have been cited to demonstrate nuclear exclusion and a punctate cytoplasmic staining, which is suggested to indicate localization to intracellular vesicles [69, 111-113, 143, 212, 323, 324]. This hypothesis is derived from reports that HERC1 associates with intracellular membranes, localization is modified during treatment with brefeldin A (a fungal metabolite which effects dissociation of the Golgi apparatus), and binds to the clathrin heavy chain of clathrin not contained in clathrin-coated vesicles [323, 324]. Additionally, HERC1 associates with several small GTPases with known roles in membrane trafficking (ARF and Rab family GTPases), and is recruited to sites of active actin polymerization following activation of the small GTPase ARF6 [111, 112, 323, 324].

Further suggestion that HERC family proteins may be involved in subcellular vesicle trafficking comes from study of mice demonstrating *rjs* syndrome (for runty, jerky, sterile, also called *jdf2*). This syndrome may be induced solely by defects in the HERC2 gene [169, 212, 318, 409], which may be linked to Prader-Willi syndrome in humans [269]. Mice with *rjs* syndrome demonstrate jerky gait, neuromuscular tremors, and reduced growth, as well as male sterility and increased juvenile mortality, and frequently, abnormal pigmentation. The developmental and neurological deficits may be explained by dysfunction of hypothalamic neurosecretory neurons and degeneration of their axons, which has been reported, suggesting that HERC2 may play a key role in vesicle trafficking in neurons [171], while the male sterility is accounted for by defects in acrosome formation and spermatid development [132, 171].

Interestingly, all HERC family proteins have been demonstrated to have greater expression in the testes (with the exception of HERC3) and brain (typically higher in foetal brain than adult), as compared to other organs [143, 212, 324]. Additionally, HERC3 is also suggested to play a role in intracellular membrane trafficking, as it has been found to colocalize with ARF proteins, Rab5, and β-COP, though not to colocalize with the Golgi vesicle-associated protein GMP_T1 or the lysosomal marker LIMP-II [69].

1.3.2 RCC1-Like Domains

Regulator of chromosome condensation 1 (RCC1) was identified as the temperature-sensitive mutant protein responsible for the phenotype of the baby hamster kidney cell line tsBN2 [272, 273, 279]. Upon shift to a non-permissive temperature, these cells demonstrate a cell cycle block prior to entry into S phase, or if already past the G1-S phase restriction point demonstrate precocious chromosome condensation and initiation of mitosis despite incomplete DNA replication [272, 279]. Subsequently, RCC1 has been shown to bind histones H2A and H2B [268, 280], as well as Ran, small GTPase [26]. RCC1 acts to activate Ran by displacing GDP from the enzyme and allowing the recruitment of GTP, acting as a guanine nucleotide exchange factor (GEF) [25, 315]. The regulated modulation of Ran by RCC1 is critical for numerous cellular processes, including nuclear import and export of macromolecules, entry into mitosis, mitotic spindle regulation, and nuclear envelope formation (reviewed in [258, 310]).

Recently, the structure of RCC1 has been solved and its mechanism of GEF function reported [8, 315, 316]. RCC1 folds to form a seven-bladed β-propeller; each blade is encoded by a characteristic repeat of 50-68 amino acids [16, 315, 316]. These characteristic repeats are found in 18 identified human proteins, while the functional significance of lesser numbers of repeats is not yet known [16]. Subsequent to the identification of RCC1-like domains (RLDs) in other proteins, their ability to function as GEFs has been queried to determine whether this function is characteristic of the domain. Support for this hypothesis is currently weak: the first RLD (RLD1) of HERC1 has been shown to catalyze

GDP dissociation (but interestingly, inhibit GTP association) from the small GTPases ARF1, Rab3a, and Rab5 [111, 324] and the RLD of *Arabidopsis thaliana* PRAF appears to catalyze the release of GDP from the *Arabidopsis thaliana* GTPases Rab8a and (to a lesser extent) Rab11c [164], while the *Drosophila melanogaster* protein Claret suggestively preferentially binds to nucleotide-free *Drosophila melanogaster* Rab-RP1 (which has homology to the human GTPases Rab32 and Rab38, which also interact with Claret), though it remains to be demonstrated whether the RLDs are directly involved in this [233]. Another RLD-containing protein, human alsin, is known to function as a GEF for the GTPases Rac1 and Rab5, though the GEF function is mediated through another domain while the RLD has no known function [389].

More likely, it seems, is that RLDs mediate protein-protein interactions. Credence is lent to this hypothesis from evidence that numerous protein interactions (including those of RCC1 to histones and Ran) are mediated through RLDs; the RLD2 of HERC1 is known to mediate the interaction of HERC1 with the heavy chain of clathrin [323], the RLD2 of PAM (protein associated with c-myc) interacts with and strongly inhibits type V adenylyl cyclase [110], while RPGR (a protein associated with X-linked retinitis pigmentosa) interacts with both RPGRIP and cGMP phosphodiesterase δ subunit through its RLD [34, 222, 320]. Nercc1, a kinase with a RLD shown to have a role in mitotic progression, provides another interesting example of RLD interactions [321]. Nercc1 preferentially interacts with GDP-bound Ran via its RLD (similar to RCC1), however Nercc1 lacks several catalytic residues in its RLD that are essential for RCC1's GEF function, perhaps hinting that Nercc1 may function as a competitive inhibitor of RCC1 [8, 321]. Additionally, the RLD of Nercc1 (carboxy-terminal) was shown to interact with the amino-terminal kinase domain, with data suggesting that this interaction functions to inhibit kinase activity [321].

An interesting correlation exists in the fact that a large number of RLD-interacting proteins identified (thus far) demonstrate nucleotide-binding capability and appear to be modulated by interaction with the RLD, such as type V adenylyl cyclase by PAM, Ran by RCC1, cGMP phosphodiesterase by RPGR, the Nercc1 kinase domain by the protein's own RLD, as well as ARF and Rab family GTPases by HERC1 [110, 111, 222, 321, 324]. Further characterization of RLD-containing proteins and protein interactions with this domain will be of great interest in elucidating its function(s).

1.3.3 Protein Modification by Ubiquitin or Ubiquitin- Like Proteins

Covalent modification of proteins is a critical mechanism to modulate the structure, interactions, and function of proteins, as well as modulating protein stability. Ubiquitin, a 76 amino acid polypeptide highly conserved throughout eukaryotes, is the prototypical member of a family of small protein modifiers [166, 181, 182, 278, 291, 299]. Ubiquitin may be covalently liked to the epsilon amino group of lysine residues on protein targets through the ubiquitin conjugation pathway [120]. While initially characterized as a mechanism for targeting proteins for proteolytic degradation by the 26S proteasome [54, 59, 140], it is now clear that protein modification by ubiquitin may have alternatively modes of function in DNA repair [147, 374], ribosomal function [373], modulation of transcription factors [174], endocytosis of membrane proteins [141], and initiation of the inflammatory response [75].

The ubiquitin conjugation pathway appears to be composed of three (though four in some cases) distinct enzymatic reactions (Figure 1.3.3). Ubiquitin is initially produced as a precursor peptide which is matured through the proteolytic cleavage of terminal amino acids to expose two terminal glycine residues (G75 and G76) [166]. Following its maturation, ubiquitin may be activated through conjugation of the carboxy terminus of ubiquitin G76 to the acceptor cysteine of the ubiquitin activating enzyme (UAE, or enzyme 1 (E1)) in an ATP-dependent process [130, 131]. The high-energy ubiquitin thioester may then be transferred to a ubiquitin conjugating enzyme (UBC, or enzyme 2 (E2)) [66-68, 379, 422]. A ubiquitin ligase (UBL, or enzyme 3 (E3)) interacts with ubiquitin conjugating enzymes as well as substrates to catalyze ubiquitination. It is the interaction of specific ubiquitin conjugating enzymes with cognate ubiquitin ligases that provides substrate specificity and allows the catalytic cleavage of the high-energy ubiquitin thioester bond and the conjugation of the G76 carboxyl group to epsilon amino group of a lysine residue of a substrate (forming an amide bond) [54, 120].

Thus far, three distinct families of E3 enzymes have been identified: those containing RING finger domains, U-box domains, and HECT domains [135, 299, 319]. It is thought that most ubiquitin ligases can catalyze the formation of polyubiquitin chains, most commonly formed by the ligation of subsequent ubiquitin G76 carboxyl groups to the epsilon amino group of lysine 48 (K48) of the previously-ligated ubiquitin (though ubiquitin-ubiquitin ligation may be catalyzed at alternative lysine residues by some ubiquitin ligases [54, 75, 147, 373, 374, 431]. However, in some instances it appears that following the initial ligation of ubiquitin to a substrate protein, the formation of polyubiquitin chains may be mediated by a ubiquitin elongation enzyme (UEE, or enzyme 4 (E4)) [187].

For ubiquitin, and all ubiquitin-like proteins characterized thus far, a single activating enzyme exists. While for ubiquitin the activating enzyme is composed of a single protein, Uba1, the activating enzymes for at least several ubiquitin-like proteins appear to be heterodimeric [79, 121, 122]. In the ubiquitin conjugation pathway, multiple ubiquitin conjugating enzymes have been identified, all of which bear a highly conserved and characteristic core domain [165, 167, 299, 300, 422]. While most ubiquitin conjugating enzymes are functional alone, a subfamily termed ubiquitin E2 variant (UEV) proteins lack the ubiquitin thioester-acceptor cysteine and require heterodimerization with a conventional ubiquitin conjugating enzyme, as exemplified by Uev1A-UbcH13 in humans [75]. It is of great interest that unlike conventional E2-E3 interactions which catalyze the formation of K48-linked polyubiquitin chains, Uev1A-UbcH13 in conjunction with the ubiquitin ligase TRAF6 catalyzes the formation of K63-linked polyubiquitin chains (which appear not to target proteins for proteasomal degradation) [75]. Unlike the ubiquitin conjugation system, the pathways involved in conjugation of ubiquitin-like molecules appear (thus far) to utilize a single E2 enzyme [80, 118, 122, 385, 438]. However, there is evidence suggesting that these systems may also utilize specific substrate consensus sequences for modification, thereby necessitating few (or perhaps only one) E2-E3 interactions for specificity [145, 166].

While a single ubiquitin activating enzyme and small number of ubiquitin conjugating enzymes are encoded in humans, hundreds of putative ubiquitin ligases have already been identified. As the ubiquitin ligase, in conjunction with its cognate E2(s), provides the specificity for substrate ubiquitination, the great diversity of E3 enzymes is required to allow for the specific and timely regulation of protein ubiquitination. Despite this difference, the pathways and mechanisms for modification by ubiquitin-like proteins (*e.g.,* SUMO/sentrin, NEDD8/Rub1, and ISG15/UCRP) appear to be entirely analogous, with the conjugation pathways for ubiquitin-like proteins composed of E1, E2, and E3 enzymes. The function of protein modification by ubiquitin-like proteins remains to be fully elucidated, but appears to modulate protein function and/or trafficking as opposed to targeting proteins for proteolytic degradation [181, 237, 416].

1.3.3.1 HECT Domains

Through efforts to elucidate the mechanism by which the E6 protein of oncogenic human papilloma viruses effected the inactivation and degradation of p53, a novel protein was identified. Directly interacting with papilloma virus E6 protein and essential for the degradation of p53, the protein was subsequently titled E6-associated protein (E6-AP) [156, 346, 348]. The inactivation of p53 was shown to be mediated by the poly-ubiquitination of p53 and subsequent degradation by the proteasome [346, 348]. The approximately 350 carboxy terminal residues of E6-AP were demonstrated to bear striking similarity to the carboxy termini of many other proteins, and thereby established the HECT (homologous to E6-AP carboxy terminus) domain [155, 156]. The subsequent characterization of further HECT domain-containing proteins defined the HECT domain as a catalytic domain effecting ubiquitin ligation to target proteins [155, 411].

Unlike U-box & RING finger domains, HECT domain ubiquitin ligases accept the ubiquitin thioester to a conserved catalytic cysteine in the HECT domain and directly mediate the ligation of ubiquitin to substrate lysine residues [135, 155, 170, 347]. HECT domain ubiquitin ligases require this catalytic cysteine residue, and cannot accept ubiquitin thioesters nor catalyze ubiquitin ligation to substrate proteins if it is mutated [347]. Additionally, a single phenylalanine (or in some cases, tyrosine) residue is required within the last five amino acids of the domain [337]. HECT domains lacking this phenylalanine (or in some cases, tyrosine) residue appear to accept ubiquitin thioesters from cognate ubiquitin conjugating enzymes, but are unable to catalyze their transfer to substrate proteins [337]. The proximity of the phenylalanine residue to the carboxy terminus of the protein also appears critical to the function of the HECT domain as well, as the addition of epitope tags as short as eight amino acids appears to block E3 function [337]. Interestingly, both HERC1 and HERC2 appear to bear carboxy terminal extensions [337], though the HERC1 HECT domain has been shown to accept ubiquitin thioesters despite this extension [349]. As substrates for HERC1 and HERC2 have yet to be identified, the function of these carboxy terminal extensions remains to be elucidated. While no HECT-domain E3 enzymes for ubiquitin-like protein have been described to date, *in vitro* evidence suggests that HECT domains are able to interact with UbcH8, which appears to be the E2 enzyme for ISG15 modification, and catalyze the ligation of ISG15 to substrates of the E3 [438]. Zhao et al. note that HERC5 is a HECT domain E3 enzyme induced with ISG15 during the interferon response, and suggest that HERC5 may act as an E3 enzyme in the ISG15 conjugation pathway [439]. However, Kroismayr et al. demonstrate that HERC5-ubiquitin thioesters can be formed using UbcH5a, thereby suggesting that HERC5 functions as E3 enzyme for ubiquitin [194]. Nevertheless, as a RING finger E3 enzyme has been shown to catalyze the ligation of NEDD8 (a ubiquitin-like protein) as well as ubiquitin, indicating that such functional overlap may also occur for HECT E3 enzymes [424].

1.3.4 Function and Regulation of HERC5

HERC5 was initially identified in yeast two-hybrid studies searching for proteins which interact with cyclin E and p21 and consequently titled cyclin E-binding protein 1 (Ceb1) [253]. In this report, HERC5 was subsequently shown to interact with cyclins A, B1, D1, and E by co-immunoprecipitations and GST pulldown assays [253]. Further, the authors suggest that two cyclin-binding regions are present distal to the RCC1-like domain, and that the HERC5-cyclin interactions do not function to promote ubiquitination of the cyclins, though no evidence was provided to support these hypotheses [253]. HERC5 is capable of accepting ubiquitin thioesters from UbcH5a in ubiquitin thioester bond formation assays *in vitro*, suggesting that it is a functional E3 enzyme [194]. To date, no other interacting partners nor substrates have been identified, leaving the function of HERC5 unknown. HERC5 is hypothesized to function in cell cycle regulation, though no evidence has been yet presented to support this theory [250, 253].

Contributing to the theory that HERC5 may play a role in cell cycle regulation, HERC5 transcription appears to be linked to dysregulation of cell cycle factors. In its initial characterization, HERC5 mRNA was shown to be present in many cell lines, but not in non-transformed human diploid lung fibroblasts (WI-38 cells) [253]. Subsequently, it was shown that HERC5 mRNA was upregulated in human diploid lung fibroblasts following infection by retrovirus vectors encoding either human papilloma virus type 16 E6 protein (inactivating p53) or human papilloma virus type 16 E7 protein (inactivating pRB) [253]. The greatest upregulation was noted following infection with a retroviral vector encoding simian virus 40 (SV40, a polyoma virus) large T antigen (which inactivates both p53 and pRB), while the expression of HERC5 was not induced by infection with a control retroviral vector [253]. This finding was furthered by expression of wild-type p53 via infection with an adenoviral vector in a pRB-competent, p53-deficient cell line (MDAH041), resulting in a reduction (but not ablation) of HERC5 mRNA levels [253]. Finally, derepression of HERC5 in WI-38 non-transformed human diploid lung fibroblasts was noted following treatment of cells with either trichostatin A or sodium butyrate. Both are histone deacetylase inhibitors, which among other effects, derepress genes repressed through the interaction of pRB with E2F transcription factors [253].

In a separate study, HERC5 was identified as gene regulated in response to the potent S phase transcription factor c-myc [250]. In this study, non-transformed human umbilical vein endothelial cells were cultured and infected with an adenoviral vector encoding c-myc, or with an adenoviral vector encoding green fluorescent protein as a control [250]. Genes induced as a consequence of these transductions were identified by serial analysis of gene expression (SAGE) [250]. The regulation of HERC5 in response to ectopic c-myc expression was further validated by quantitative polymerase chain reaction (PCR), chromatin immunoprecipitation of c-myc at the HERC5 promoter, and microarray analysis [250].

Two recent papers also suggest that HERC5 is upregulated in response to expression of human telomerase (hTERT) [201, 327]. Telomerase is a cellular protein not normally expressed in somatic cells (but expressed in germ cells), but which is thought to be essential for cell immortalization (due to the apparent necessity of telomeres for genetic maintenance during replication [50, 63, 71, 209, 328]). In these studies, ectopic expression of hTERT was mediated by retroviral transduction of donor-obtained human CD4$^+$ T lymphocytes [327] or derivation of a stably-transformed cell line after transfection of a neonatal human diploid fibroblasts (MJ90 cells) with a plasmid vector containing hTERT cDNA [201]. In both studies, an enhancement of HERC5 expression (amongst other genes) was identified by microarray analysis [201, 327]. This intriguing finding may provide an explanation for the expression pattern of HERC5 in human tissues: typically low to non-detectable in tissues composed of somatic cells, while slightly higher expression was noted in ovarian tissue and much higher expression was noted for HERC5 expression in the testes [143, 253].

Paradoxically, HERC5 transcription also appears to be induced under conditions typically associated with cell cycle arrest. Three independent groups have identified HERC5 as a novel gene induced during the antiviral response

[189, 259, 376]. The first indications that HERC5 may be involved in antiviral defence were obtained from microarray analysis of host cell transcriptional response to infection with human herpes virus 1 (HHV1, or herpes simplex virus 1 (HSV1)) [259]. In this study, HERC5 was found to be significantly induced in non-transformed human diploid embryonic lung fibroblasts following infection with transcriptionally-inactivated HHV1 but not wild-type HHV1 [259], implying that wild-type HHV1 is capable of suppressing the induction of a subset of genes (many known members of the interferon and antiviral responses) including HERC5.

Following this report, infection of OVCAR4 ovarian carcinoma cells with wild-type or attenuated strains of vesicular stomatitis virus (VSV) was shown to induce a subset of genes associated with the interferon and antiviral responses, as well as HERC5 [376]. It is of note that wild-type VSV appears to induce primary antiviral genes but suppresses the transactivation of a secondary transcriptional class, while the attenuated, interferon-sensitive VSV strains used in this study induced stronger transcription of primary antiviral genes and did not block the subsequent transactivation of later kinetic classes of antiviral genes [376]. In this study, HERC5 was induced with similar kinetics (and to similar levels) as compared with well characterized primary antiviral responsive genes [376], thereby providing correlative data strongly suggesting that HERC5 is in fact a primary-transcriptional member of the antiviral response.

Quite recently, an examination of the transcriptional events correlated with West Nile virus infection also identified HERC5 as induced during viral infection [189]. In this study, a human glioblastoma cell line (A172 cells) were infected with wild-type West Nile virus and compared to uninfected cells by microarray analysis [189]. Amongst other genes involved in the antiviral and interferon responses, apoptosis, mitochondrial function, and translation, HERC5 was induced [189]. This finding was confirmed by quantitative reverse transcriptase PCR (qRT-PCR), leading to the interesting finding that the induction of HERC5 as determined by microarray analysis and qRT-PCR were quite different [189], which may suggest that (in at least this system) microarray analysis grossly under-estimates the induction of at least two genes (HERC5 and interferon-inducible transmembrane protein 1 (IFITM1)).

A recent report additionally implicates HERC5 as a novel gene induced by pro-inflammatory stimuli in endothelial cells [194]. Interestingly, cells of different anatomic origins demonstrated remarkably different inductions of HERC5 in response to lipopolysaccharide (LPS) or tumour necrosis factor α (TNFα). Human skin microvascular endothelial cells, human aortic endothelial cells, and human uterine microvascular endothelial cells potently induced HERC5 transcription in response to stimulation. In contrast, human umbilical vein endothelial cells, human smooth muscle cells, and human foreskin fibroblasts demonstrated minimal to non-detectible responses [194]. In the human microvascular endothelial cell line HM2, HERC5 was inducible by the pro-inflammatory stimuli interleukin 1β, TNFα, and LPS, but not by other cytokines such as interleukin 6, interleukin 8, interleukin 10, epithelial growth factor, vascular endothelial growth factor, or transforming growth factor β [194]. Interestingly, the induction of HERC5 in HM2 cells was largely suppressed by pre-treatment with cycloheximide [194], suggesting that maximal transactivation of HERC5 is at least partially dependent on translation of a novel transcript, and is therefore part of a secondary (or subsequent) transcriptional class of pro-inflammatory inducible genes. Complementing this finding, the authors demonstrated that ectopic over-expression of IκBα (which interacts with the pro-inflammatory transcription factor NFκB and blocks its nuclear import) in human skin microvascular endothelial cells and human uterine microvascular endothelial cells blocks transactivation of HERC5 [194]. Thus, at least one factor essential to the transactivation of HERC5 is dependent on the activation of NFκB. Interestingly, no cis elements of inflammation-associated transcription factors were found in the HERC5 promoter by bioinformatic analysis [194]. The role of HERC5 expression in response to pro-inflammatory stimuli in human microvascular endothelial cells has yet to be elucidated.

Anecdotal evidence connects HERC5 expression to several diseases, which may be related to its apparent regulation in response to transforming events and/or the antiviral response. Correlative studies on gene expression in cancerous cell types have also found interesting results with regard to HERC5. Over-expression of HERC5 was described to occur in a majority of 14 examined human pancreatic ductal adenocarcinoma cell lines, as identified by serial analysis of gene expression (SAGE) and confirmed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) [157]. Further correlation of HERC5 expression with malignant disease is provided by a study of cancerous human breast tissue obtained by biopsy [446]. In this study, HERC5 was found to be over-expressed, as assessed by SAGE, in metastatic nodal breast carcinoma, though potentially absent in normal breast epithelial cells and primary invasive breast ductal carcinoma [446]. Interestingly, numerous other genes reported to be responsive to c-myc were also reported to be upregulated in the metastatic nodal breast carcinoma tissue [250, 446], perhaps suggesting that deregulation of c-myc expression is linked to the progression of breast carcinoma to metastatic neoplastic disease. While these studies identified over-expression of HERC5 as a feature of several malignant cell types, down regulation of HERC5 was suggested to be a feature of many human hepatocellular carcinomas [428]. In this study dissected human hepatocellular carcinoma and non-tumourous control tissue from the same individuals were analysed for the dysregulation of genes located in chromosome 4q21-24 (a region frequently altered in hepatocellular carcinoma [27, 428] by qRT-PCR. No mutations in HERC5 were reported in these studies, and it remains to be resolved whether dysregulation of HERC5 occurs as consequence of malignancy or HERC5 dysregulation contributes to malignant transformation.

Another reported correlation of HERC5 over-expression is more puzzling. In a family afflicted with hereditary parkinsonism, a disease typically associated with mutation in the gene α synuclein and (perhaps consequent) cytoplasmic inclusions in glial cells and formation of Lewy bodies [397], no evidence for mutation or altered transcript splicing of α synuclein was identified [369]. Rather, in this family parkinsonism (but not postural tremor) was associated with the triplication of a region of q arm of chromosome 4 containing HERC5 (as well as α synuclein) [369]. No further correlative or causative evidence has been produced to suggest whether or how triplication of HERC5 may be related to Parkinson's disease.

1.4 Experimental Rational

The little that is known about the transcriptional regulation and protein interaction of HERC5 appears highly intriguing. Associated with molecular events which promote cell cycle dysregulation and transformation [201, 250, 253, 327], as well as innate immune events which typically arrest the cell cycle [189, 194, 210, 259, 376], characterization of HERC5 is further complicated by its apparently recent evolutionary emergence in primates [113, 143]. It is an interesting coincidence that HERC5 mRNA does not appear to be transcribed at detectible levels in U-2 OS osteosarcoma cells [253], which interestingly are at least partially responsive to interferon [259, 260], but have been shown to complement interferon-sensitive viruses [260, 427]. Thus, correlative data indicates that HERC5 may be a novel member of the antiviral response and its antiviral function may be related to the ability of this protein to bind to cyclins, though this interaction is of unknown function. The purpose of this work is to further characterize the transcriptional regulation of human HERC5 in response to stimuli associated with the antiviral response, and to examine the effects of the reported interaction of HERC5 with cyclins.

Figure 1.1.1: Activation of Innate, Intracellular Antiviral Pathways by Recognition of Pathogen-Associated Molecular Patterns Lead to the Transcriptional Activation of Responsive Genes.

Activation of innate immune signalling pathways through the recognition of pathogen-associated molecular patterns (*e.g.,* double-stranded RNA, viral glycoproteins). While many different types of pathogen-associated molecular patterns are recognized through a variety of Toll-like receptors, double-stranded RNA appears unique in the multitude of cellular receptors devoted to its detection (*i.e.,* PKR, TLR3, RIG-1, MDA-5). Following recognition of a pathogen-associated molecular pattern by a cognate receptor, signalling pathways are activated leading to the activation of several transcription factors (*e.g.,* NFκB, ATF-2, c-JUN, IRF3), consequently activating transcription of genes associated with the antiviral response.

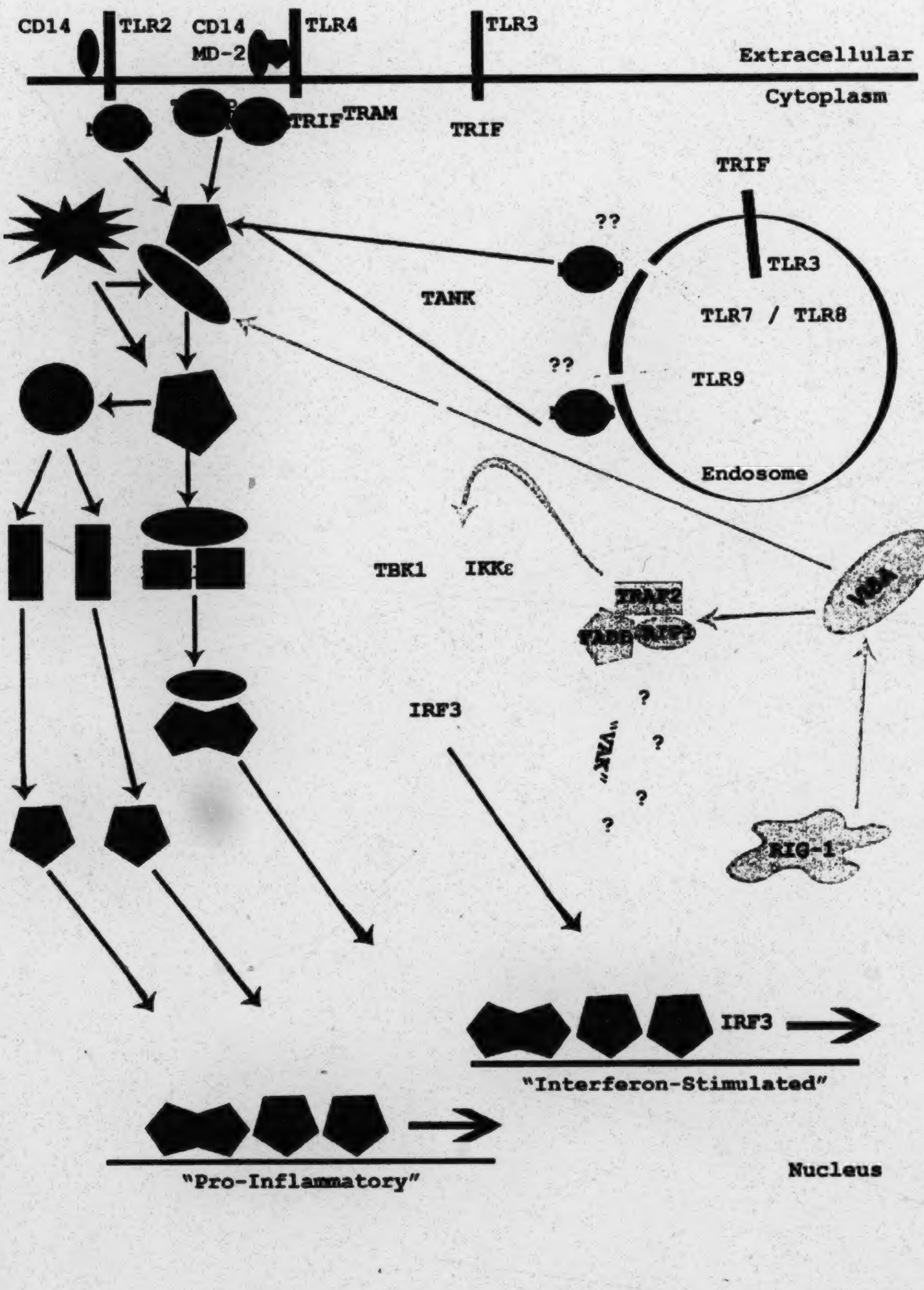

CD14
TLR2
CD14
MD-2
TLR4
TLR3
Extracellular
Cytoplasm
TRIF
TRAM
TRIF
TRIF
??
TLR3
TANK
TLR7 / TLR8
??
TLR9
Endosome
TBK1
IKKε
TRAF2
FADD
IRF3
?
"VAK"
?
?
?
RIG-1
IRF3
"Interferon-Stimulated"
"Pro-Inflammatory"
Nucleus

Figure 1.1.3: Activation of Intracellular Antiviral Response Leads to the Activation of a Transcriptional Cascade of Antiviral Gene Stimulation.

Activation of the antiviral response, either through the type I interferon receptor or through innate pathogen-associated molecular pattern recognition pathways, can result in the activation of transcription factors leading to the synthesis of antiviral gene products. Among these products, the production of type I interferons (*e.g.,* interferon β, interferon α) facillitate the preventative activation of antiviral response pathways in nearby cells, but also function to positively feedback and bolster the antiviral pathway by autocrine signalling events.

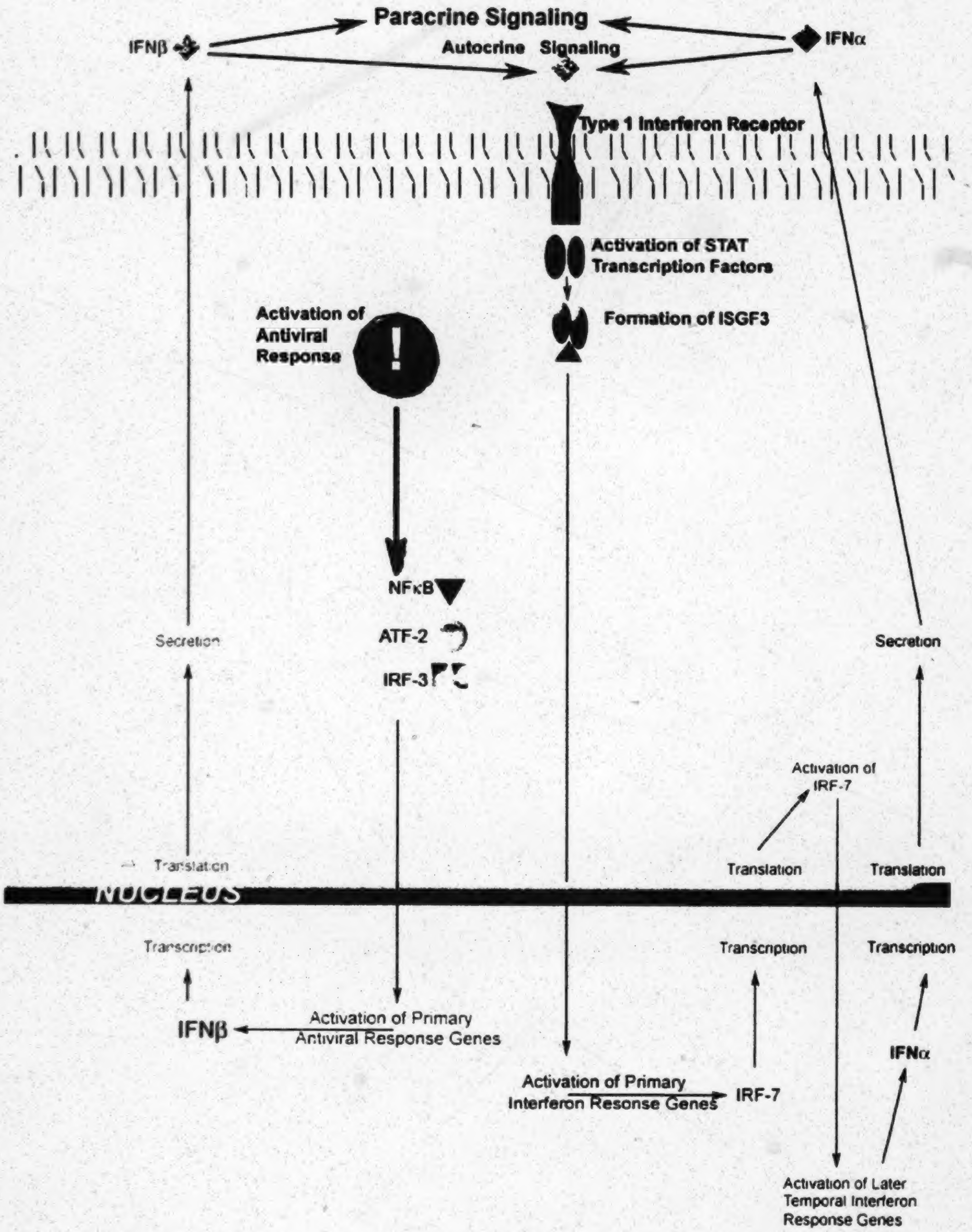

Paracrine Signaling
IFNβ
Autocrine Signaling
IFNα
Type 1 Interferon Receptor
Activation of STAT Transcription Factors
Formation of ISGF3
Activation of Antiviral Response
NFκB
ATF-2
IRF-3
Secretion
Secretion
Activation of IRF-7
Translation
Translation
Translation
NUCLEUS
Transcription
Transcription
Transcription
IFNβ
Activation of Primary Antiviral Response Genes
IFNα
Activation of Primary Interferon Resonse Genes
IRF-7
Activation of Later Temporal Interferon Response Genes

Figure 1.2.1: Cyclin-Dependent Kinase Activity in Association with Cell Cycle Phase Progression.

Eukaryotic cellular proliferation occurs through the highly regulated activation of cyclin-dependent kinsases, largely through the temporal regulation of their cognate cyclins. Activation of cyclin C-cyclin-dependent kinase 3 in response to mitogenic stimuli potentiates continued cell cycle progression following completion of mitosis, or resumption of the cell cycle in resting cells (G0). Continued mitogenic stimuli drive the transcription of D-type cyclins, which may complex with either of cyclin-dependent kinases 4 or 6 and promote cell cycle progression through G1 phase. Activation of cyclin-dependent kinase 2 by cyclin E promotes progression past the restriction point, marking commitment to completing cell division, and leads to progression into S phase. Upon entry into S phase, newly produced cyclin A may bind and activate cyclin-dependent kinase 2 and promote DNA synthesis. Upon completion of S phase, cyclin A preferentially activates cyclin-dependent kinase 1 and marks a shift into G2 phase, allowing the cell to prepare for mitotic division. Initiation and progression through mitosis is coordinated through the effects of cyclin B in complex with cyclin-dependent kinase 1, with the final events completing mitosis leading to the degradation of mitotic cyclins and consequent inhibition of cyclin-dependent kinases. Should no mitogenic stimuli be present, cells may exit the cell cycle and enter a resting state (G0 phase), or if mitogenic stimuli are present and sufficient to trigger the transcription of C and D cyclins, cell cycle progression may continue.

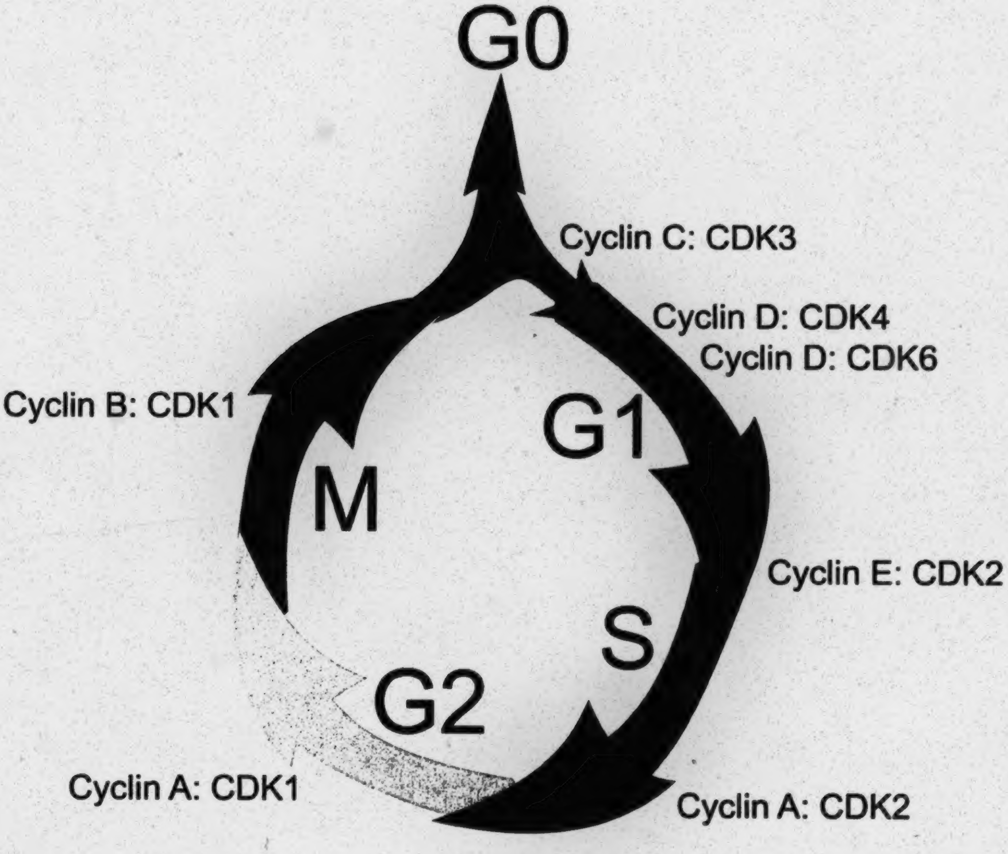
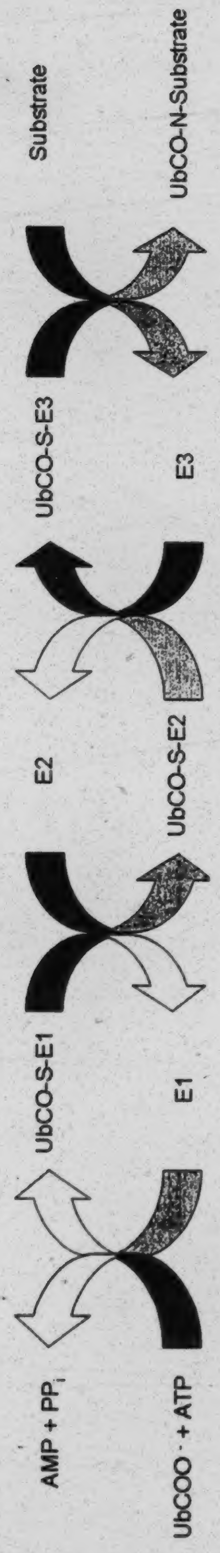

G0
Cyclin C: CDK3
Cyclin D: CDK4
Cyclin D: CDK6
Cyclin B: CDK1
G1
M
Cyclin E: CDK2
S
G2
Cyclin A: CDK1
Cyclin A: CDK2

Figure 1.3.3: The Ubiquitin Conjugation Pathway.

Ubiquitination of target proteins is mediated through the ubiquitin conjugation pathway. Initially, the carboxy terminus of ubiquitin is covalently coupled through a thioester linkage to ubiquitin activating enzyme (E1) in an ATP-dependent reaction. Cognate interaction of the ubiquitin-charged E1 enzyme with a ubiquitin conjugating enzyme (E2) facilitates transfer of the high-energey ubiquitin thioester to the E2 enzyme. Docking of a ubiquitin-charged E2 enzyme on a HECT domain-containing ubiquitin ligase (E3) facilitates transfer of the high energy ubiquitin thioester to the E3 enzyme. The HECT E3 ligases may then transfer the ubiquitin moiety to a lysine residue on a bound substrate protein, with the carboxy terminus of ubiquitin forming an amide linkage with the lysine residue on the target protein. The further conjugation of ubiquitin residues, forming poly-ubiquitin chains, may be further facilitated by the E3 enzyme when its cognate E2 enzyme is present and charged with ubiquitin, or may be facilitated through the activity of a ubiquitin elongation enzyme (E4).


AMP + PP$_i$
UbCOO - + ATP
UbCO-S-E1
E1
E2
UbCO-S-E2
UbCO-S-E3
E3
Substrate
UbCO-N-Substrate

Chapter 2: Materials and Methods

2.1. Bacterial Cell Culture, Plasmids, and Molecular Cloning

2.1.1. Propagation of Plasmids

To confirm and ensure that plasmids are constructed and maintained as intended, bacterial cultures bearing plasmid DNA with antibiotic resistance markers were screened for specific restriction endonuclease sites to verify their identity. Plasmids were maintained and amplified in the *Escherichia coli (E. coli)* strain DH5α^{TM} (genotype: F$^-$ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1, Invitrogen, Burlington, ON, Canada). Transformation of *E. coli* was achieved by combining 40 µl of electro-competent DH5α^{TM} with approximately 50 ng of plasmid DNA and pulsing at 1.8 kV, 25 µF, 200 Ω using a Gene Pulser II electroporation apparatus (Bio-Rad, Hercules, CA, USA). Immediately following electroporation, the bacteria were transferred into 1 mL of SOC medium (20 mg/mL bacto-tryptone (BD Difco, Sparks, MD, USA), 5 mg/mL bacto-yeast extract (BD Difco), 20 mM glucose, 85.5 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride), and incubated at 37°C for one hour with shaking at ~225 rpm. Aliquots of the electroporated cells were then spread-plated on Luria-Bertani agar plates (10 mg/mL bacto-tryptone, 5 mg/mL yeast extract, 171 mM sodium chloride, 1.5% agarose, mixture from BD Difco) containing suitable antimicrobials (e.g., ampicillin (110 µg/ml), chloramphenicol (25 µg/ml), and/or kanamycin (50 µg/ml) (all from Sigma-Aldrich, Oakville, ON, Canada) and cultured at 37°C. When colonies appeared on the plates, individual colonies were picked (using sterile micropipette tips) and inoculated into Luria-Bertani broth (1.0% bacto-tryptone, 0.5% yeast extract, 171 mM sodium chloride, BD Difco) supplemented with appropriate antimicrobials. Cultures were grown at 37°C overnight, while shaken at approximately 225 rpm. Plasmids were purified from bacterial cultures using QIAGEN Plasmid Maxi Kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions. Plasmid concentrations and purity were estimated spectrophotometrically (Ultrospec 3000 spectrophotometer, Amersham

Biosciences, Baie d'Urfe, QC, Canada), examining the photoabsorbance of aqueous dilutions of samples in matched quartz cuvettes at 260 nm, 280nm, and 320 nm. Plasmids were stored at -20°C.

2.1.2. Screening of Plasmids by Restriction Digestion

Plasmids were isolated from one millilitre aliquots of bacterial cultures using a QIAquick Spin Miniprep Kit (Qiagen), according to the manufacturer's instructions. Aliquots of the miniprep (approximately 10% of the miniprep yield) were then supplemented with 10 units (total) of restriction enzyme(s) and suitable buffer (as per the manufacturer's recommendations (Invitrogen, New England Biolabs, Ipswich, MA, USA, or Fermentas, Burlington, ON, Canada) in a total reaction volume of 10 µL. Enzymatic digestion of the miniprep occurred for one hour at 37°C, unless otherwise suggested by the enzyme's manufacturer. Upon completion of the one hour incubation, 2 µL of Gel Loading Buffer (50% (v/v) glycerol, 0.2% bromophenol blue, 0.2% xylene cyanol, 1 mM tetrasodium ethylenediaminetetraacetate (EDTA)) were added to the digestion reactions, and the total contents were loaded into the wells of a 1% agarose-TAE gel (40 mM tris(hydroxymethyl)aminomethane (Tris) acetate, 1 mM EDTA, 12.6 mM ethidium bromide, pH 8.3), with 750 ng of *Bst*EII-digested bacteriophage λ DNA (New England Biolabs) loaded into a separate well as a molecular weight marker. Agarose-TAE gels were submerged in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3), and electrophoresed at 110 V until sufficient resolution of the molecular weight marker was attained. Gels were digitally photographed under ultraviolet light (UVP White/UV Transilluminator, DIAMED Laboratory Supplies, Mississauga, ON, Canada, Scion Image v1.59 software for Apple Macintosh, Scion Corporation, Frederick, MD, USA). Experimentally obtained DNA band patterns were compared to the theoretical patterns predicted (by sequence analysis) using pDRAW32 v1.1.87 software for Microsoft Windows (AcaClone Software, www.acalone.com) to verify plasmid identity.

2.1.3. Site-Directed Mutagenesis

Alterations to existing DNA sequence, either to incorporate novel restriction cleavage sites or to alter the coding sequence of a gene, were achieved by site-directed mutagenesis (Quikchange XL Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). Briefly, a polymerase chain reaction (PCR) was performed using a complementary pair of mutagenic primers and *PfuTurbo* DNA polymerase (Stratagene), as per the manufacturer's protocol. Following PCR, the input plasmid DNA (methylated) was degraded by supplementation of the reaction with 10 Units of *Dpn*I (a restriction endonuclease specific for the sequence G 5-Methyl-A T C), and further incubation at 37°C for one hour. An aliquot of this *Dpn*I-digested, mutagenized plasmid was then used to transform *E. coli* strain XL10-Gold® by heat-shock (genotype: TetR Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*qZΔ*M15* Tn*10* (TetR) Amy CamR], Stratagene), as per manufacturer's instructions. Following heat-shock transformation, 0.5 mL of SOC medium was added to the reaction and incubated for one hour at 37°C. Aliquots were then spread on Luria-Bertani agar plates supplemented with 110 µg/mL ampicillin, and grown overnight at 37°C. Colonies were picked, propagated, and screened as described in sections 2.1.1 and 2.1.2. Following screening, the open reading frame-of-interest was sequenced (Department of Biochemistry DNA Core Services Facility, University of Alberta, Edmonton, AB, Canada).

2.1.4. Sub-Cloning by Restriction Digestion

To identify potential cloning strategies, the nucleotide sequence of plasmids were input into pDraw32 (AcaClone Software). Using pDraw32, the locations of all present restriction cleavage sites were identified. Approximately 2 µg of each vector and insert-bearing plasmid were digested with one unit of restriction enzyme (total) per 100 ng DNA per microlitre reaction volume. Restriction enzymes were purchased from New England Biolabs, Invitrogen, or Fermentas. For multiplex digestions, the reaction buffers supplied by the aforementioned companies were compared for their compatibility with the restriction enzymes chosen: the reaction buffer which resulted in the greatest activity for all enzymes used (with no activity less then 75% of maximal) was used. Digestion of plasmid DNA occurred at 37°C for one hour, unless otherwise recommended by the enzyme's manufacturer.

2.1.4.1. Purification of DNA Fragments

Following restriction digest, the reactions were loaded into a 1% agarose-TAE gel and electrophoresed, imaged, and analyzed as described in section 2.1.2. Subsequently, gels were dissected under ultraviolet transillumination to isolate the desired DNA fragments. To elute the DNA fragments from the agarose slivers, a QIAquick Gel Extraction Kit (Qiagen) was used, as per manufacturer's instructions.

2.1.5. DNA Ligation

Following purification of DNA fragments, insert and vector were combined in an approximate 10:1 molar ratio with 10 units of bacteriophage T4 DNA ligase in 1x T4 ligase buffer (Invitrogen), at a total reaction volume of 20μL. Ligation reactions were incubated overnight at room temperature. In order to remove the salts inherent in T4 ligase buffer, 10 μL of the ligation was placed on a 0.05 μm membrane (Millipore, Nepean, ON, Canada) and dialyzed against one litre of 10 mM Tris, pH 8.0 for 15 minutes. The dialysed ligation was then electroporated in *E. coli* DH5α, propagated, and screened as described in sections 2.1.1 through 2.1.2. Ligations of DNA at sites included in the open-reading frame were sequenced (DNA Core Services Facility, University of Alberta) across the ligation site to ensure the integrity of the product plasmid.

2.1.6. Oligonucleotide Linker Insertion

To introduce novel sequences into plasmids, double-stranded oligonucleotides were inserted into plasmids at specific restriction sites. To anneal complementary oligonucleotides, 2 nmol of each oligonucleotide (or 4 nmol for palindromic oligonucleotides) were diluted in 10 mM Tris, 100 mM sodium chloride, pH 7.4 in a total reaction volume of 100 µL. The oligonucleotides were denatured by heating the solution at 95°C for five minutes, and annealing facilitated by removal from heat and allowing the solution to cool to room temperature, resulting in a 20 µM solution of the double-stranded oligonucleotide. The plasmid to be modified and the double-stranded oligonucleotide were combined at molar ratios between 1:50 and 1:500, followed by ligation, propagation, and screening as described in sections 2.1.5, 2.1.1, and 2.1.2, respectively. Positive clones were verified by sequencing across the insertion site (Department of Biochemsitry DNA Core Services Facility, University of Alberta) to ensure that the plasmid product contained only a single copy of the insert and was present in the correct location.

2.1.7. HERC5 Expression Plasmids

2.1.7.1. pCS2+CEB1-Full and pCDNA3-CEB1-Full

Two mammalian-expression vectors, pCS2+CEB1-Full [253] and pCDNA3-CEB1-Full, each encoding the full-length cDNA of human HERC5 mRNA were graciously provided by Dr. Motoaki Ohtsubo (Kurume University, Kurume, Fukuoka, Japan). Both plasmids contain the HERC5 cDNA insert between the *Bam*HI and *Xho*I sites of their parent vector, pCS2+ [333, 398] and pCDNA3 (Invitrogen), respectively.

2.1.7.2. pGQ01

To facilitate downstream manipulations, an *Nco*I restriction site was introduced at the initiation codon of HERC5 in pCS2+CEB1-Full by site-directed mutagenesis, using the mutagenic primers CEB1-MUT(-) (sequence: CGA CCT CCG CTC CAT GGC CGC TTT GCG GG) and CEB1-MUT(+) (sequence: CCC GCA AAG CGG CCA TGG AGC GGA GGT CG). The entire HERC5 open reading frame was sequenced to confirm the integrity of the product using the following primers:

SP6: GAT TTA GGT GAC ACT ATA G
SEQ CEB1 A: TGT GGA CAA CTA GGC CTG GG
SEQ CEB1 B: ATC TCC TGA TTG TCT AAC TGG
SEQ CEB1 C: TGA CTG TGG ACG CTT CAG
SEQ CEB1 D: GAT GAT GAA GGT GAT AAC TTT GAG G
SEQ CEB1 E: GTG GAG ATT ACC ATT CTC TTG C
SEQ CEB1 F: GCA CTG ACA TGT TTC AGT GTC

Through the course of this sequencing, it was discovered that pCS2+CEB1-Full (and subsequently pGQ01) contained a highly repetitive GTT motif downstream of the termination codon (Figure 3.1.2). This repetitive GTT motif is absent in pCDNA3-CEB1-Full, which bears the *Xho*I site 12 nucleotides downstream of the termination codon, but is present in the 3' untranslated region (3' UTR) of several HERC5 cDNA sequences, including the NCBI reference sequence (accessions: AB027289, AY337518, NM_016323), as well as both the Celera and open-source human genome sequences (accessions NT_086651 and NT_016354, respectively). Thus, the GTT repetitive motif is a real feature of HERC5 mRNA, with as yet unknown significance.

2.1.7.3. pGQ02

The 3' UTR of a transcript can have marked effects on mRNA stability and translation [46, 74, 97, 357, 364]. As such, it seemed prudent to remove the GTT-repeat motif so as to limit the effect of potential mRNA-instability elements on HERC5 over-expression. The 3' UTR of pCDNA3-CEB1-Full does not contain the repetitive GTT motif. To these ends, the 3069 base-pair *Bam*HI-*Eco*47III fragment of pGQ01 was inserted in place of the *Bam*HI-*Eco*47III fragment of pCDNA3-CEB1-Full, thereby generating pGQ02.

2.1.7.4. pGQ03

To increase the number of flanking restriction cleavage sites, and thereby facilitate downstream sub-cloning, the 3094 base-pair *Cfr*I-*Xba*I fragment of pGQ02 was inserted between the *Not*I and *Xba*I sites of pCDNA3.1+ (Invitrogen) to create pGQ03.

2.1.7.5. pGQ04

Due to the presence of several *Nco*I sites in the pCDNA3 and pCDNA3.1+ vectors (Invitrogen), an alternative vector was needed to facilitate manipulation of the plasmid at this restriction site. The bacterial-glutathione-S-transferase-fusion expression vector pGEX-4T3 (Amersham Biosciences) was chosen as a suitable vector to these ends [370]. To incorporate a single *Nco*I site into the multiple cloning site of pGEX-4T3, the vector was linearized by digestion with *Bam*HI and the palindromic double-stranded oligonucleotide BamNcoBam (sequence: GAT CCA TG) was ligated into the vector to create pGEX-4T3-GQ. The 3085 base-pair *Nco*I-*Xho*I fragment of pGQ02 was then inserted between the *Nco*I and *Xho*I sites of pGEX-4T3-GQ, producing pGQ04.

2.1.7.6. pGQ05 and pGQ06

As no satisfactory antibodies are available to detect HERC5 protein, the construction of a mammalian-expression vector encoding an epitope-tagged form of HERC5 was needed to facilitate studies of protein localization. To these ends, the FLAG epitope (sequence: DYKDDDK) was selected due to its purported low background, as well as availability of reagents. To create a vector to express the FLAG epitope at the immediate amino-terminus of HERC5, a double-stranded oligonucleotide (encoding the FLAG epitope, +FLAG-BamNco: GAT CCA TCG ATG GAC TAC AAA GAC GAT GAC GAC AAG CT, and –FLAG-BamNco: CAT GAG CTT GTC GTC ATC GTC TTT GTA GTC CAT CGA TG) was combined with the *Nco*I-*Xho*I fragment of pGQ04 (3085 base-pairs) inserted between the *Bam*HI and *Xho*I sites of pGEX-4T3-GQ (resulting in the FLAG-HERC5 open reading frame being out-of-frame from the glutathione-S-transferase open reading frame), thereby generating pGQ05. To transfer the FLAG-HERC5 open reading frame into a mammalian-expression vector, the *Bam*HI-*Not*I fragment of pGQ05 (3129 base-pairs) was inserted between the *Bam*HI and *Not*I sites of pCDNA3.1+ (Invitrogen), producing pGQ06.

2.1.7.7. pGQ07 and pGQ08

In order to create stably-transfected tetracycline-inducible mammalian cells lines, the mammalian-expression vector pTRE2pur (BD Clontech) was utilized. This vector contains the minimal cytomegalovirus immediate-early promoter downstream of the Tetracycline Response Element (TRE), resulting in transcriptional regulation in response to tetracycline and derivatives (such as doxycycline) [123]. The plasmid also encodes puromycin N-acetyl-transferase (driven by the simian-virus 40 early promoter), conferring resistance to puromycin and allowing efficient selection in eukaryotic cells. The *Bam*HI-*Not*I fragments of pGQ03 (3095 base-pairs) and pGQ06 (3129 base-pairs) were inserted between the *Bam*HI and *Not*I sites of pTRE2pur (BD Clontech), resulting in the plasmids pGQ07 and pGQ08, respectively.

2.1.7.8. pGQ09 and pGQ10

To facilitate biochemical analysis of potential ubiquitin E3 ligase activity of HERC5, a 5'-truncated cDNA of HERC5 (encoding amino-acids 344 through 1024) was generated by digesting pGQ03 with *Pml*I and *Xho*I, and purifying the resulting 2084 base-pair fragment. This fragment was inserted between the *Sma*I and *Xho*I site of pGEX-4T3 (Amersham Biosciences), resulting in a glutathione-S-transferase-HERC5$_{344\text{-}1024}$ bacterial expression vector named pGQ09 [370]. pGQ10 was created by subcloning the *Bam*HI-*Xho*I fragment of pGQ09 (2099 base-pairs) into the *Bam*HI and *Sal*I sites of pQE30 (Qiagen), a bacterial-expression fusion vector, resulting in the fusion of a hexa-histidine tag to the amino-terminal of HERC5$_{344\text{-}1024}$.

2.1.7.9. pGQ11 and pGQ12

To elucidate the effects of the HECT domain on HERC5 localization and potential protein functions, a mammalian-expression vector encoding a catalytically-inactive variant of the HECT domain was required. To these ends, the codon encoding the (putative) catalytic cysteine of HERC5's HECT domain was identified by bioinformatic analysis [114, 155, 367], and mutated (by site-directed mutagenesis) to encode alanine (C994A mutation) using the mutagenic primers +C994A (sequence: CCT ATA GCA CTG ACA GCT TTC AGT GTC CTC TC CTC) and -C994A (sequence: GAG GAA GAG GAC ACT GAA AGC TGT CAG TGC TCT TAT AGG). The incorporation of this mutation into pGQ05 resulted in pGQ11.

2.1.7.10. pGQ14 and pGQ15

To cross-verify the localization of HERC5 protein determined by use of pGQ06 and concomitantly rule out interference caused by addition of the amino-terminal FLAG epitope, epitope-tagging of the carboxy-terminus of HERC5 could be used as a control. To these ends, the 747 base-pair *Eco*47III-*Xho*I fragment of pEGFP-C1 (BD Clontech) was inserted in place of the *Eco*47III-*Xho*I fragments (12 base-pairs and 49 base-pair) of pGQ06. The resultant plasmid, named pGQ15, encoded a carboxy-terminal truncation of HERC5 (HERC5$_{1\text{-}1013}$) with an amino-terminal FLAG epitope tag and a carboxy-terminal fusion to a variant of the green fluorescent protein (GFP) of *Aequorea victoria*. To generate a variant of pGQ15 lacking the amino-terminal FLAG epitope tag, the 1403 base-pair *Nhe*I-*Hpa*I fragment of pGQ03 was inserted in place of the 1437 base-pair *Nhe*I-*Hpa*I fragment of pGQ15, thereby generating pGQ14.

2.1.7.11. pGQ16

Further to the goals described in section 2.1.7.8, a vector for the constitutive expression of HERC5$_{C994A}$ in mammalian cells, pGQ16, was created. To these ends, the *Bam*HI-*Not*I fragment of pGQ11 (3129 base-pairs) was inserted between the *Bam*HI and *Not*I sites of pCDNA3.1+ (Invitrogen).

2.1.7.12. pGQ18

In order to determine if the RCC1-like domain of HERC5 affected protein localization or function, a vector encoding an amino-terminal truncation of HERC5 was desired. To accomplish this, the *Pml*I- *Xba*I fragment of pGQ14 (2800 base-pairs) was ligated between then *Eco*RV and *Xba*I sites of pCDNA3.1+ (Invitrogen). The resultant plasmid, pGQ18, encodes a fusion protein consisting of HERC5$_{348-1013}$ with a carboxy-terminal enhanced-GFP tag.

2.1.7.13. pGQ22

To compliment pGQ18, a second vector encoding an amino-terminal truncation of HERC5 was created. pGQ22 was created by removing the *Hpa*I-*Xba*I fragment of pGQ03 (1738 base-pairs) and inserting it into p3xFLAG-CMV10 (Sigma-Aldrich). As such, pGQ22 encodes a triple-FLAG epitope tag fused to amino-truncated HERC5 (HERC5$_{455-1024}$).

2.1.7.14. pGQ23 and pGQ25

The region of HERC5 lying between the RCC1-like domain and the HECT domain putatively contains at least one cyclin-binding site [253]. To determine whether this region of the protein affected HERC5 localization or function, a vector encoding a HERC5 variant containing an internal 174 amino-acid truncation (HERC5$_{1-485,\ 660-1024}$) was created. To accomplish this, pGQ05 was digested with *Hin*DIII to remove a 522 base-pair fragment. Ligation of the remaining pGQ05 fragment created pGQ23. To transfer the open reading frame into a mammalian-expression vector, the *Bam*HI-*Xho*I fragment of pGQ23 (2601 base-pairs) was inserted between the *Bam*HI and *Xho*I sites of pCDNA3.1+ (Invitrogen) to create pGQ25.

2.1.8. Human Cyclin Expression Plasmids

2.1.8.1. pRcCMV-cyclin A

The mammalian-expression plasmid pRcCMV-cyclin A [142] was obtained from Dr. Luis Schang (University of Alberta). This plasmid contains a cDNA copy of human cyclin A mRNA, driven by the cytomegalovirus immediate early promoter.

2.1.8.2. pRcCMV-cycB1

Ectopic expression of human cyclin B1 was facilitated using pRcCMV-cycB1, a mammalian-expression vector graciously provided by Dr. Peter Whyte (McMaster University, Hamilton, ON, Canada). The vector was constructed using the pRcCMV vector backbone [142] and the BstXI fragment of human cyclin B1 cDNA [302].

2.1.8.3. pRcCMV-cycD1

For the ectopic expression of human cyclin D1, pRcCMV-cycD was obtained from Dr. Peter Whyte (McMaster University). This plasmid also utilizes the pRcCMV vector backbone [142], into which the *Eco*RI-*Hin*DIII fragment of human PRAD1 cDNA was inserted [261].

2.1.8.4. pGQ13

To determine if HERC5 expression affected the localization of cyclin E, a mammalian-expression vector encoding human cyclin E was employed. The bacterial-expression vector, pBSKS-cyclin E, which contains a cDNA copy of human cyclin E mRNA, was created by Susan Lacy and was a kind gift from Dr. Peter Whyte (McMaster University). The cyclin E open reading frame was removed from pBSKS-cyclin E by restriction digest with *Eco*RI and *Xho*I to generate a 1711 base-pair fragment, which was sub-cloned into the *Eco*RI and *Xho*I sites of pCDNA3.1+, creating pGQ13.

2.1.9. Other Mammalian-Expression Plasmids

2.1.9.1. pBB14

The Us9-GFP fusion protein is a type II membrane-anchored protein [40] that is quantitatively retained in mammalian cells after fixation and permeablization [175, 176], thus making it a suitable marker for transfected-cell selection during cell cycle analysis. To these ends, pBB14 was graciously provided by Dr. Lynn Enquist (Princeton University, Princeton, NJ, USA). pBB14 encodes the Us9 protein of pseudorabies virus, fused at its carboxy terminus to a variant of the green fluorescent protein (GFP) of *Aequorea Victoria*, driven by the cytomegalovirus immediate-early promoter.

2.1.9.2. p3xFLAG-CMV10-Dectin 2

As a control for the FLAG epitope, the vector p3xFLAG-CMV10-Dec-2 was utilized (Joshua Christianson, Ostergaard Laboratory, University of Alberta). The vector contains the cDNA for the extracellular domain of murine Dectin 2α inserted into the p3xFLAG-CMV10 vector backbone (Sigma Aldrich).

2.1.9.3. pEGFP-C1

The mammalian-expression of the enhanced-green fluorescent protein (GFP) of *Aequorea Victoria*, using pEGFP-C1 (BD Clontech), served as a control for transient transfection and non-specific protein localization in mammalian cells.

2.2. Mammalian Cell Culture

2.2.1. Mammalian Cell Types

U-2 OS human (female) osteosarcoma cells [17], HeLa human (female) cervical adenocarcinoma cells, Vero African green monkey kidney cells, and HEL 299 primary human (male) embryonic lung (HEL) fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA). 293A and 293T transformed human (female) embryonic kidney cell lines were generous gifts from Drs. John F. Elliott and Deborah Burshtyn, respectively (University of Alberta). A549 human (male) lung carcinoma cells were a kind gift from Dr. Mary Hitt (University of Alberta). UTA6 cells, a derivative of U-2 OS cells which express the tetracycline-repressed transcriptional activator fusion protein[123], were graciously obtained from Dr. David Litchfield [406] (University of Western Ontario, London, ON, Canada), originally derived by Dr. Christoph Englert [95].

HEL cells were cultured up to 20 passages, while all other cell lines were cultured up to 50 passages before thawing fresh cells.

2.2.2. Maintenance of Mammalian Cells

Cells were maintained in 150 cm^2 tissue culture flasks (Corning, Acton, MA, USA) with 20 mL of complete medium, consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Cansera, Etobicoke, ON, Canada), 1 mM sodium pyruvate (Invitrogen), with 50 units/ml penicillin G and 5 μg/ml streptomycin (PenStrep) (Invitrogen). UTA6 cells were additionally supplemented with 500 μg/ml G418 (Geneticin®, Invitrogen). Cells were cultured at 37°C in a humidified atmosphere supplemented to 5% CO_2. Cell cultures were passaged upon or prior to reaching confluence. Briefly, cells were rinsed with phosphate-buffered saline (PBS, 10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4), followed by proteolytic detachment of cells using 2.5 g/L trypsin and 0.38 g/L EDTA (1x Trypsin-EDTA, Invitrogen) at room temperature. Upon complete detachment of cells, further proteolysis was inhibited by the addition of 5 mL of complete medium. The concentration of cells was then estimated by direct count (using a haemocytometer) of an aliquot of cell suspension, and cells were seeded at desired densities into culture vessels with suitable volumes of complete medium.

In some experiments, cytoplasmic translation was inhibited by the inclusion of 100 μM cycloheximide (Sigma-Aldrich) in the medium. Cycloheximide was added 30 minutes prior to any other treatments, and was continually present in the medium of such samples, to ensure the complete inhibition of translation in response to any factors subsequently added. Such factors and their final (medium) concentrations include: 1,000 International Units/mL human alpha-interferon from leukocytes (IFNα, Sigma-Aldrich), 1,000 International Units/mL recombinant human gamma-interferon (IFNγ, Cell Sciences, Canton, MA, USA), 1 ng/mL recombinant human interleukin-1 beta (IL1β, Research Diagnostics Inc., Flanders, NJ, USA), 1 ng/mL tumour necrosis factor alpha (TNFα, Sigma-Aldrich), 25 μg/mL double-stranded polyriboinosinic-polyribocytidific acid (Poly I:C, Sigma-Aldrich), and 1 μg/mL lipopolysaccharide (LPS, Sigma-Aldrich).

2.3. Virus Strains and Growth Conditions

2.3.1. Amplification of Virus Stock

Human herpesvirus 1 (herpes simplex virus type 1, HSV-1) strain KOS 1.1 is a wild type virus [153]. To obtain a personal preparation, Vero cells were cultured to confluence in 150 cm^2 tissue culture flasks and subsequently infected with approximately 0.01 plaque-forming units (PFU) of HSV-1 KOS1.1 per cell in 5 mL DMEM. Infection was allowed to proceed for one hour at 37°C, with rocking every 20 minutes. Following this infection incubation, the initial inoculum was aspirated and replaced with 15 mL of medium 199 (Invitrogen, Burlington, ON, Canada), supplemented with 10% (v/v) FBS and PenStrep. Infected cells were incubated at 37°C in a humidified atmosphere supplemented to 5% CO_2.

When the vast majority of cells demonstrated severe cytopathic effects (*i.e.*, were rounded and easily lost adherence to the tissue flask upon shaking), typically four days post-infection, cells remaining attached were mechanically sheared from the tissue culture flask and the total cell suspension was collected. Intact cells and sufficiently dense cell debris were separated from the medium by centrifugation at approximately 2,000 x g for 10 minutes at 4°C (Beckman GS-6R centrifuge, GH-3.8 rotor, Beckman Coulter, Mississauga, ON, Canada). The supernatant was discarded, while the pellet was resuspended in 1 mL of DMEM. Cell lysis was effected through three cycles of freezing (–80°C) and thawing (37°C) the pellet suspension. Subsequently, the cell pellet was chilled on ice and then subjected to three rounds of sonication (20 seconds at setting 7 using a Fischer 550 Sonic Dismembrator (Fisher Scientific, Ottawa, ON, Canada) fitted with a cup-horn sonication chamber. Each round of sonication was followed by a one minute rest on ice to prevent thermal denaturation of proteins. Following sonication, the suspension was centrifuged at approximately 2,000 x g for 10 minutes at 4°C. The supernatant was collected and stored as 100 µL aliquots in 1.5 mL cryovials (Nalge Nunc International, Rochester, NY, USA).

2.3.2. Titration of Viral Preparations

Serial dilutions of virus preparations were titrated by plaque assay on both Vero and U-2 OS cells. Cells were seeded into 12-well tissue culture plates (Corning) with complete medium. Upon reaching confluence, monolayers of cells were washed with PBS. Serial ten-fold dilutions of the virus preparation were performed in duplicate, with DMEM as the diluent. Wells were infected using 0.25 mL of each viral dilution, and incubated at 37°C in a humidified atmosphere supplemented to 5% CO_2 for one hour, with rocking every 20 minutes. Subsequent to this incubation, the viral inoculum was replaced by 1 mL of DMEM supplemented by 1% heat-inactivated human immune serum (ICN, Montreal, QC, Canada), 9% FBS, and PenStrep. Cells were incubated in the aforementioned conditions until plaques were apparent by visual inspection. Medium was then aspirated and cells fixed using methanol (-20°C) for ten minutes, followed by staining of cells overnight with modified Giemsa stain (Sigma-Aldrich). Grids were drawn on the bottom of the tissue culture plates and plaques enumerated by direct count facilitated by light microscopy.

2.3.3. Viral Infection of Mammalian Cells

Prior to infection, replicate tissue culture plates or wells were seeded with cells in complete medium, and grown to confluence. Upon reaching confluence, one of the replicate plates/wells was rinsed with PBS followed by proteolytic detachment of cells using 1x Trypsin-EDTA (Invitrogen). Upon complete detachment of cells, proteolysis was inhibited using complete medium. An aliquot of the cell suspension was then counted using a haemocytometer, and from this the number of cells in a confluent monolayer could be estimated. The remaining cell monolayers were rinsed with PBS, and infected at the desired number of viral plaque-forming units per cell (multiplicity of infection, MOI), using DMEM as the diluent (typically at one quarter the normal volume of medium).

Infection proceed at 37°C in a humidified atmosphere supplemented to 5% CO_2 for one hour, with rocking every 20 minutes. Subsequent to this incubation, the viral inoculum was replaced by complete medium, further incubated as required.

2.4. Qualitative Analysis of Cellular Ribonucleic Acids

2.4.1. Ribonucleic Acid Extraction

Monolayers of cells were grown to confluence in complete medium. Upon reaching confluence, medium was aspirated and replaced with 1 mL TRIZOL® reagent (Invitrogen). The lysate was collected using a cell-scraper and incubated at room temperature for five minutes to allow dissociation of nucleoprotein complexes. Ribonucleic acids (RNA) were extracted from the lysate by the addition of 0.2 ml chloroform, manually shaken for 20 seconds, and centrifuged at 12,000 x g (Eppendorf centrifuge 5417C, Eppendorf, Westbury, NY, USA) for 15 minutes in a 4°C cold-room. The aqueous phase was transferred to an RNase-free microfuge tube (Rose Scientific Ltd., Edmonton, AB, Canada), 0.5 ml isopropanol was added, and samples were incubated for 15 minutes at room temperature. RNA was pelleted by centrifugation for 10 minutes under the previously mentioned conditions, followed by aspiration of the supernatant. The RNA pellet was resuspended in 0.5 ml 70% (v/v) ethanol and centrifuged for 5 minutes under the aforementioned conditions. The supernatant was discarded and the pellet was resuspended in 30 μl of diethyl pyrocarbonate-treated (DEPC) water [105, 198] . Ribonucleic acid concentrations and purity were estimated spectrophotometrically (Ultrospec 3000 spectrophotometer). The aqueous RNA samples were stored at -80°C.

2.4.2. Formaldehyde-Agarose Gel Electrophoresis

Desired amounts of each RNA sample were brought to equal concentration, and mixed in a 1:2 ratio with RNA loading buffer (22.5 μM MOPS, 10.4 μM sodium acetate, 0.4 μM EDTA, 20% (v/v) formaldehyde, 60% (v/v) formamide, pH 7.0). To approximate the size of RNA species, 2 μg of RNA Millennium™ Size Markers (Ambion, Austin, TX, USA) were also mixed with

RNA loading buffer. Marker and samples were loaded into the wells of a formaldehyde-agarose gel (1.8% (v/v) formaldehyde, 18 mM MOPS, 8.3 mM sodium acetate, 0.5 mM EDTA, 12.6 mM ethidium bromide, 1% agarose gel), submerged in formaldehyde-agarose gel running buffer (18 mM MOPS, 8.3 mM sodium acetate, 0.5 mM EDTA, pH 7.0). Gels were subjected to 100 V until sufficient resolution of marker RNA was obtained.

2.4.3. Probes

Template DNA for ^{32}P-labeled probe was either amplified by PCR or isolated by restriction digest. The following sets of plasmids and primers (all from Department of Biochemistry DNA Core Services Facility, University of Alberta) or restriction enzymes were used to make the probes for this investigation:

ICP0 probe: Plasmid: pSHZ [264]
Restriction enzymes: *Bam*H I and *Xho* I
431 bp probe fragment

TK probe: Plasmid: pTK173 [402]
Restriction enzymes: *Sac* I and *Sma* I
662 bp probe fragment

ISG 56k probe: Plasmid: IMAGE clone 325364 (Invitrogen)
Primer 1: CCC AGT CAC GAC GAC GTT GTA AAA CG
Primer 2: AGC GGA TAA CAA TTT CAC ACA GG
~1300 bp probe fragment

HERC5 probe: Plasmid: IMAGE clone 37942 (Invitrogen)
Primer 1: CCC AGT CAC GAC GAC GTT GTA AAA CG
Primer 2: AGC GGA TAA CAA TTT CAC ACA GG
~1600 bp probe fragment

Glyceraldehyde-3-Phosphate Dehydrogenase probe:

Oligonucleotide: TTG ACT CCG ACC TTC ACC TTC CCC AT

26 base oligonucleotide probe

To label cDNA fragments with ^{32}P, random-primed labelling of cytidine residues was used. Briefly, 2 µg of random primers (hexadeoxynucleotides, DNA Core Services Facility, University of Alberta) were added to 250 ng of template DNA in 35 µl water (total volume), and denatured at 100°C for 3 min. The denatured reaction mixture was chilled for 2 min on ice. The mixture was supplemented to: 44 mM Tris, 4.4 mM magnesium chloride, 9.1 mM 2-mercaptoethanol, 17.6 µM deoxyadenosine triphosphate, 17.6 µM deoxyguanosine triphosphate, 17.6 µM deoxythymidine triphosphate, 181 mM HEPES, 0.4 mg/mL bovine serum albumin (BSA, New England BioLabs). To this, 5 µL of α-^{32}P deoxycytosine triphosphate (Amersham Biosciences) and 5 units of DNA polymerase I Klenow fragment (Invitrogen) were added (total volume 50 µL), allowing incorporation of label for 30 minutes at 37°C. Following this incubation, incorporation was inhibited by the addition of an equal volume of TE buffer (10 mM Tris, 1mM EDTA, pH 7.0), and extracted with phenol:chloroform:isoamyl alcohol (25:24:1 v/v, Invitrogen). The aqueous layer was applied to a NICK™ column (Amersham Biosciences) in order to remove nucleic acids less than 20 bases in length, with the labelled probe eluted in 500 µL of TE buffer. The radioactivity of probes was assayed using CytoScint™ Scintillation fluid (ICN) with a Beckman LS 6500 Liquid Scintillation Counter (Beckman Coulter).

2.4.4. Northern Blotting

Upon completion of electrophoresis, RNA was transferred to a nylon membrane (GeneScreen Plus, NEN Life Science Products, Boston, MA, USA) by capillary diffusion in 10x SSC buffer (150 mM sodium citrate, 1.5 M sodium chloride). The RNA was cross-linked to the nylon membrane by ultraviolet irradiation (auto-cross-linked twice, Stratalinker 2400, Stratagene). Marker and ribosomal RNA (rRNA) bands were located under ultraviolet light and marked on the nylon membrane using an ultra-fine Sharpie permanent marker. The membranes were then pre-hybridized with 5 mL ExpressHyb™ hybridization solution (BD Biosciences Clontech, Mountain View, CA, USA) for 30 minutes at 68°C under rotation (H1 16000 hybridization incubator, Tyler Research Instruments, Edmonton, AB, Canada).

Pre-hybridization solution was replaced with 10 mL ExpressHyb™, supplemented with 10^7 counts per minute of ^{32}P-labelled probe cDNA. The membrane was incubated with the probe for 1 hour in the previously-mentioned hybridization condition. Subsequently, the hybridization solution was decanted and the blot was washed with three changes of Wash Buffer #1 (1.5 mM sodium citrate, 15 mM sodium chloride, 3.5 mM sodium dodecyl sulphate (SDS)) over 30 minutes while rotated at room temperature. The membrane was then washed with three changes of Wash Buffer #2 (30 mM sodium citrate, 300 mM sodium chloride, 1.75 mM SDS) over 20 minutes while rotated at 50°C. Membranes were then wrapped in Saran Wrap (SC Johnson, Racine, WI, USA), and stored at -20°C. The decay of ^{32}P was detected by autoradiography using Kodak BioMax MS film (Kodak, Toronto, ON, Canada).

2.5. Qualitative Detection of Cellular Proteins

2.5.1. Cell Lysis and Polyacrylamide Gel Electrophoresis

Monolayers of cells were washed with PBS, and then lysed in 250 µl SDS-PAGE lysis buffer (10mM Tris-HCl, 70 mM SDS, 74.6 µM bromophenol blue, 200 mM 1,4-dithio-DL-threitol, 25% glycerol, pH 6.8). Lysates were boiled at 100°C for 5 min to ensure denaturation and reduction prior to loading an aliquot onto a sodium dodecyl sulphate-polyacrylamide gel (37.5:1 acrylamide to bis-acrylamide) along with 10 µl Precision Plus Protein All Blue molecular weight markers (Bio-Rad). Samples were electrophoresed for 1-2 hrs at 150 V in sodium dodecyl sulphate-polyacrylamide gel electrophoresis buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS, pH 8.3).

2.5.2. Western Blotting

Upon completion of electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using a Trans-Blot protein transfer apparatus with plate electrodes and cooling coil (Bio-Rad) with transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) at 1.5 A for approximately one hour. Following protein transfer, membranes were blocked by submersion in Western Blocking Solution (25 mM Tris, 150 mM sodium chloride, 0.1 % (v/v) Tween-20 (Fischer Scientific), 5% (m/v) skim milk powder (Nestle, North York, ON, Canada), pH 8.0) overnight at 4°C.

The following dilutions of primary antibodies were used: monoclonal mouse IgG anti-β-actin (Sigma-Aldrich) 1:5,000, Anti-FLAG M2 monoclonal mouse IgG anti-FLAG epitope (Sigma-Aldrich) 1:1,000, Anti-FLAG polyclonal rabbit IgG anti-FLAG epitope (Sigma-Aldrich) 1:1,000, monoclonal mouse IgG anti-human cyclin E (clone HE12, Upstate, Waltham, MA, USA) 1:1,000, polyclonal rabbit IgG anti-human cyclin A (SC-751, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:100. Antibodies were diluted in Western Blocking Solution and incubated with the membrane for one hour at room temperature with rocking. Following this incubation, the membrane was washed three times in Tween 20-Tris-buffer saline (TTBS, 25 mM Tris, 150 mM sodium chloride, 0.1% (v/v) Tween 20 (Fischer Scientific), pH 8.0) over one hour, with shaking. Following these washes, appropriate secondary antibodies (polyclonal goat IgG anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) 1:5000, or polyclonal goat IgG anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) 1:5000) were diluted in Western Blocking Solution and incubated with the membrane for one hour at room temperature with rocking. Following this incubation, the membrane was again washed three times in TTBS over one hour, with shaking. Detection was facilitated by use of ECL+ Western Blotting Detection Reagents (Amersham Biosciences) and Fuji Super RX X-ray film (FUJIFILM Medical Systems, Stamford, CT, USA).

2.6. Transfection of Mammalian Cells

Cells were seeded in 12 well tissue culture plates (Corning) containing 18 mm, #1 thickness round coverslips (Fischer Scientific) at approximately 2 x 10^5 cells/well with complete medium.

2.6.1. Transient Transfection Using FuGENE 6

Three microlitres of FuGENE 6 transfection reagent (Roche Applied Science, Laval, QC, Canada) were added to 50 µL of Opti-MEM I medium (Invitrogen) and gently agitated (by hand) to mix. One microgram of plasmid DNA was added to the solution and gently agitated (by hand) to mix, and incubated at room temperature for 20 minutes to allow FuGENE 6-DNA complexes to form. Following this incubation, the solution was added drop-wise to a well, followed by gentle agitation of the plate to facilitate dispersion of the transfection complexes. Cells were incubated at 37°C in a humidified atmosphere supplemented to 5% CO_2 until fixed.

2.6.2. Stable Transfection Using FuGENE 6

UTA6 cells were plated at 4 x 10^6 cells per 10 cm tissue culture dish and transfected using FuGENE 6 (2 µg plasmid:6 µL FuGENE 6) as described in section 2.5.1.1. Four hours post-transfection, the cell medium was supplemented to 5 µg/mL doxycycline (Fluka, Buchs, Switzerland), and cells returned to incubation for 12 hours. At this time, cells were trypsinized and divided into five 10 cm^2 dishes with selection medium (complete medium supplemented to contain 500 µg/mL Geneticin (Invitrogen), 5 µg/mL doxycycline (Fluka), and 1 µg/mL puromycin (Sigma-Aldrich)). Cells were washed and selection medium was replaced every second day to ensure removal of dead cells and maintenance of selection. Transfection of plasmids lacking the puromycin resistance marker functioned as a positive control for the efficacy of the selection medium, with all cells in the non-puromycin-resistant control plates dead within four days. Eleven days after the initial selection, sixteen distinct colonies of viable cells were isolated using cloning cylinders, trypsinized, and replated into a 24-well tissue culture plate. These individual colonies were maintained in selection medium and continually passaged to generate several 150 cm^2 tissue culture flasks.

These stable cell lines were trypsinized, with cells numbers estimated by haemocytometer count, and pelleted by centrifugation at 750 x g for 5 minutes at 4°C (Beckman GS-6R centrifuge, GH-3.8 rotor, Beckman Coulter). The supernatant was aspirated and the cells resuspended in ice-cold 5% (v/v) dimethyl sulphoxide 95% FBS at an approximate cell density of 5 x 10^6 cells/mL. One millilitre aliquots of cells were transferred into chilled 1.5 mL cryovials (Nalge Nunc International), stored at -80°C overnight, and then stored submerged in liquid nitrogen indefinitely. Cell lines were screened for expression as described in section 2.5. Of the sixteen clones selected, the clones titled G and L demonstrated the greatest induction upon the removal of doxycycline from the media (assayed as described in section 2.5) with the lowest basal expression of FLAG-HERC5.

2.6.3. Transient Transfection Using Lipofectamine 2000

Three microlitres of Lipofectamine 2000 transfection reagent (Roche Invitrogen) and one microlitre of plasmid DNA were added to separate 50 µL aliquots of Opti-MEM I medium (Invitrogen) and gently agitated to mix. The two aliquots were then combined, gently agitated to mix, and incubated at room temperature for 20 minutes to allow Lipofectamine 2000-DNA complexes to form. Following this incubation, the solution was added drop-wise to a well, followed by gentle agitation of the plate to facilitate dispersion of the transfection complexes. Cells were incubated at 37°C in a humidified atmosphere supplemented to 5% CO_2 for 3 hours, rinsed with PBS, and then returned to incubation with fresh complete medium until fixed.

2.6.4. Transient Transfection Using ExGEN 500

One microgram of plasmid DNA was added to 100 µL of 150 mM sodium chloride and briefly, gently agitated to mix. Three microlitres of ExGen 500 transfection reagent (Fermentas, Burlington, ON, Canada) were added to the solution and vortexed for ten seconds to mix. The solution was then incubated at room temperature for 20 minutes to allow ExGen 500-DNA complexes to form. Following this incubation, the solution was added drop-wise to a well, followed by gentle agitation of the plate to facilitate dispersion of the transfection complexes. Cells were incubated at 37ºC in a humidified atmosphere supplemented to 5% CO_2 for 3 hours, rinsed with PBS, and then returned to incubation with fresh complete medium until fixed.

2.7. Indirect Immunofluorescence Assay

Indirect immunofluorescence was carried out based on the method described by Boutell and Everett [32]. Cells were incubated for 18-24 hours (plus additional treatments, if performed), rinsed with PBS, and then fixed with Fix Solution (58.4 mM sucrose, 5 % (v/v) formaldehyde, PBS) for 10 minutes at room temperature. Fix Solution was then aspirated and wells washed twice with PBS. Cells were subsequently permeablized by incubation in Permeablization Solution (292 mM sucrose, 0.6% (v/v) Nonidet P-40 (BDH Laboratory Supplies, Poole, England), PBS) for 10 minutes at room temperature. Cells were then rinsed twice in Immunofluorescence Blocking Solution (1% FBS in PBS) and incubated for 10 minutes at room temperature to allow blocking.

Coverslips containing fixed, permeablized cells were placed cells-down on a 50 µL aliquot of primary antibody, diluted in Immunofluorescence Blocking Solution, for one hour at room temperature. The following dilutions of primary antibodies were used: monoclonal mouse IgG anti-β-actin (Sigma-Aldrich) 1:2,000, Anti-FLAG M2 monoclonal mouse IgG anti-FLAG epitope (Sigma-Aldrich) 1:250, Anti-FLAG polyclonal rabbit IgG anti-FLAG epitope (Sigma-Aldrich) 1:250, polyclonal rabbit IgG anti-human cyclin A (SC-751, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:100, polyclonal rabbit IgG anti-human cyclin B1 (SC-752, Santa Cruz Biotechnology) 1:100, monoclonal mouse IgG anti-cyclin D1 (SC-6281, Santa Cruz Biotechnology) 1:100, monoclonal mouse IgG anti-cyclin D3 (SC-6283, Santa Cruz Biotechnology) 1:100, monoclonal mouse IgG anti-human cyclin E (clone HE12, Upstate, Waltham, MA, USA) 1:250. Coverslips were then washed four times, cells-up, with Immunofluorescence Blocking Solution to remove non-specifically-bound antibodies. Following these washes, appropriate secondary antibodies (polyclonal goat IgG anti-mouse IgG conjugated to either Alexa Fluor 488 or Alexa Fluor 546 (Invitrogen) 1:250, or polyclonal goat IgG anti-rabbit IgG conjugated to either Alexa Fluor 488 or Alexa Fluor 546 (Invitrogen) 1:250) were diluted in Immunofluorescence Blocking Solution. Coverslips were placed cells-down on 50 µL aliquots thereof and incubated for one hour at room temperature in the dark. Coverslips were then washed four times, cells-up, with Immunofluorescence Blocking Solution, followed by dipping in distilled, deionized water to remove non-specifically-bound antibodies. Coverslips were mounted on standard glass microscope slides (Fischer Scientific) using 20µl Vectashield mounting medium (Vector Laboratories, Burlington, ON, Canada), and secured with clear nail polish.

To better visualize the nuclear-cytoplasmic boundaries, the nuclei of cells were counterstained using DNA-intercalating fluorophores. To facilitate multiplexing of the immunofluorescence assay, several different nucleic acid stains with different spectral properties were used. When used, propidium iodide (Invitrogen) was included in the final distilled, deionized water rinse of coverslips at a concentration of 1.5 mM. Alternatively, DAPI (4'-6-diamidino-2-phenylindole dihydrochloride, Invitrogen) was included in the mounting medium at an approximate concentration of 4 µM. As a third alternative, after fixation of cells, DRAQ5 (Biostatus Ltd, Shepshed, Leicestershire, England) was diluted into PBS (1:1,000) and incubated with coverslips for 15 minutes at 37°C.

Slides were examined using a Zeiss LSM 510 Laser Scanning Microscope system with four lasers giving excitation lines at 351 nm (DAPI), 488 nm (Alexa Fluor 488), 543 nm (Alexa Fluor 546), and 633 (DRAQ5), and using a 40x oil-immersion objective lens.

2.7.1. Detection of Apoptotic Cells

To determine if transfected cells were apoptotic, TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling) was used to detect fragmentation of nuclear DNA [30, 72, 115, 119, 255, 355]. To ensure a positive control for apoptosis, two cell-coated coverslips (one transfected, the other non-transfected, 19 hours post-transfection) were placed in a tissue culture dish with PBS and subjected to 500 μJ/cm^2 of ultraviolet irradiation (StrataLinker 2400, Stratagene) [31]. The medium for all coverslips was then replaced with fresh complete medium, followed by further incubation. Tissue culture plates were examined periodically (by light microscopy) to determine the effects of the ultraviolet irradiation. When typical features of apoptosis, including condensed nuclei and faint cell-cell borders, were evident in the irradiated cells (approximately 7 hours post-irradiation), cells were fixed with ice-cold 50% ethanol 50% glacial acetic acid for 2 minutes. To remove the fixative, cells were washed twice with PBS before placing coverslips cells-down on 50 μL aliquots of TUNEL enzyme-label reaction mixture (containing fluorescein-labelled deoxyuridine triphosphate, Roche Applied Science), as per the manufacturer's directions. As a negative control, one coverslip was incubated with TUNEL label alone. The TUNEL reaction was allowed to proceed for one hour at room temperature in the dark. Coverslips were then washed with PBS three times in 15 minutes, and subsequently incubated with Immunofluorescence Blocking Solution for 15 minutes before continuing with indirect immunofluorescence.

2.8. Cell Cycle Analysis

2.8.1. Permeablization and Staining of Non-Fixed Cells

Due to potential confounding artefacts, including loss of GFP fluorescence due to denaturation of GFP, a protocol for the cell cycle analysis of non-fixed cells was used [296]. Following all treatments, cells were trypsinized from tissue culture dishes/plates and cells collected by centrifugation at approximately 750 x g for 5 minutes at 4°C (Beckman GS-6R centrifuge, GH-3.8 rotor, Beckman

Coulter). The supernatant was aspirated and the pellet resuspended in 1 mL of PBS, followed by centrifugation under the aforementioned conditions. The supernatant was aspirated and the pellet resuspended in 500 µL Ghost Buffer (20 mM HEPES, 1 mM EGTA (ethylene glycol-bis(2-aminoethyl ether)-N, N, N', N'-tetra-acetic acid), 160 mM sodium chloride, pH 7.2) at 4°C, followed by addition of 500 µL of 0.04% digitonin (Sigma-Aldrich) in Ghost Buffer. The cell suspension was briefly, gently vortexed, incubated on ice for 5 minutes, and then centrifuged under the aforementioned conditions. The supernatant was discarded and the pellet resuspended in 1 mL of 200 µg/mL RNase A, 15 mM propidium iodide, Ghost Buffer and incubated at room temperature, in the dark for 30 minutes. Samples were subsequently stored at 4°C (for up to 6 hours) before analysis. Propidium iodide intercalation, which is proportional to the DNA content of the cell, was quantified using a Becton-Dickson FACScan pyrometer equipped with a cell-doublet discrimination module (BD Biosciences) and analyzed using CellQuest and ModFitLT v2.0 software (BD Biosciences). Approximately 15,000 cells per sample were analyzed by forward and side scatter of light, as well as by the amplitude and width of detected FL2 channel fluorescence (propidium iodide) to discriminate and exclude cell doublets and cell debris. Only the single-cell population was analyzed for cell cycle distribution.

Chapter 3: Results

3.1. Characteristics of HERC5 cDNA and Protein

3.1.1. Sequence Verification of Human HERC5 cDNA

Upon the creation of pGQ01, a human HERC5 cDNA expression vector derived from pCS2+CEB1-Full [253], the open reading frame was sequenced to ensure the integrity of the created plasmid. The compiled sequence of the pGQ01 HERC5 cDNA was then aligned to two published human HERC5 cDNA sequences (AB027289 [253] and AY337518 [194]). Additionally, the HERC5 mRNA primary structure was modeled, using Model Maker (NCBI, National Center for Biotechnology Information, www.ncbi.nlm.nih.org), from the NCBI Reference and Celera human genome sequences. Through this process, the sequence of the pGQ01 HERC5 cDNA diverged from the reported description of the parent plasmid [253] and the published human HERC5 cDNAs, though the HERC5 cDNA sequence of pGQ01 closely matches the modeled HERC5 mRNA sequences generated from the NCBI Reference and Celera human genome sequences (Figure 3.1.1). It is of interest to note that the HERC5 cDNA encoded by pGQ01 differs from the Celera and NCBI Reference human genome-derived sequences at only one or two sites, respectively. Both of these deviations are recognized nucleotide polymorphisms, with an average allele frequency of 0.469 for the A1492G transition, and unreported frequency for the T2358C transition (Reference SNP, NCBI, www.ncbi.nlm.nih.gov/snp/).

3.1.2. Identification of a Repetitive Motif in the 3' Untranslated-Region of HERC5 mRNA

Unexpectedly, sequencing downstream of the HERC5 termination codon in pGQ01 identified a long series of GTT repeats. These repeats were not part of the pCS2+ vector [333, 398], nor part of the intended parental vector, pCS2+CEB1-Full [253]. Nevertheless, sequencing of pCS2+CEB1-Full identified an identical tract in the 3' untranslated region of the HERC5 cDNA. To determine if this feature was endogenous to HERC5mRNA, the 3' untranslated regions of two published human HERC5 cDNA sequences (AB027289 [253] and AY337518 [194]), two modeled HERC5 cDNAs (NCBI Reference and Celera human genome sequences, Model Maker, NCBI), and the HERC5 cDNA inserts of pGQ01 and pGQ03 were aligned (Figure 3.1.2). The GTT repetitive motif contains 12 GTT repeats, beginning 30 nucleotides downstream from the HERC5 termination codon, and is disrupted only once (after the third repeat) by an ATC insertion. The GTT repetitive motif is present in all of the sequences aligned, with the exception of pGQ03, and has no known function. However, when a MegaBlast search (www.ncbi.nlm.nih.gov/blast/) is performed on the 3' untranslated region of HERC5, a large number of highly homologous sequences are presented, suggesting that the HERC5 mRNA 3' untranslated region is not unique but rather may have functional significance. As the 3' untranslated region of mRNAs may modulate mRNA stability and efficiency of translation, it seemed prudent to remove the as-yet uncharacterized 3' untranslated region of HERC5 in favour of bovine growth hormone polyadenylation sequence and other sequences contained downstream of the multiple cloning region of pCDNA3.1+ (Invitrogen).

3.1.3. Predicted Primary Protein Structure of Human HERC5

From the aligned cDNA representations of HERC5 mRNA (Figure 3.1.1), the putative amino acid sequences of HERC5 were aligned (Figure 3.1.3). While containing several divergences from the predicted protein sequences from the cDNAs AB027289 and AY337518, the protein sequence predicted by the pGQ01 HERC5 cDNA closely matches those predicted by the HERC5 mRNAs modeled from the NCBI Reference and Celera human genome sequences. It is currently unknown if the A1492G nucleotide polymorphism, conferring the M498V mutation, affects HERC5 functional interactions. As the HERC5 cDNA encoded by pGQ01 bears fewer divergences from the modeled human genome-derived sequences than either of the experimentally-obtained cDNA sequences (AB027289 and AY337518), it may provide the most accurate vehicle for further characterization of HERC5 function.

3.2. Transcriptional Activation of HERC5

3.2.1. Human HERC5 is an Interferon α-Inducible Gene

HERC5 transcription has been previously demonstrated to be induced during infection of non-transformed fibroblasts with defective viruses [259, 376], infection of a transformed cell line in response to West Nile virus [189], as well as by interferon α stimulation of a melanoma cell line [210]. To determine whether interferon α could induce HERC5 expression in a variety of cell types, cells were treated with 1000 IU/mL of interferon α. Total mRNA was then harvested at various times post-stimulation. Expression of HERC5 was compared to that of the interferon-stimulated gene ISG 56k by Northern blot analysis (Figure 3.2.1). Equal loading of RNA was verified by ethidium bromide staining of the ribosomal RNA (Figure 3.2.1). As demonstrated by Mitsui et al. [253], HEK293 and HeLa cells constitutively express HERC5, while neither U-2 OS cell nor a non-transformed human embryonic lung fibroblast cell type (HEL cells) demonstrate detectable levels. This experiment additionally demonstrates that HERC5 transcription is induced by interferon α-stimulation in HEK293, HeLa, and HEL cells, with kinetics similar to those of seen with induction of ISG 56k transcription. Of great interest, though ISG 56k gene induction was rapidly and strongly induced by interferon α-stimulation of U-2 OS cells, HERC5 mRNA remained at undetectable levels. Thus, HERC5 is an interferon-inducible gene in many cell lines and non-transformed cell types, though U-2 OS cells are defective for its induction in response to interferon α.

3.2.2. Human HERC5 is a Primary Interferon α-Inducible Gene

Rapid induction of HERC5 transcription has been demonstrated in response to defective viruses [376] and interferon α-stimulation (Figure 3.2.1). However, it remained ambiguous whether HERC5 was a primary interferon-stimulated gene (*i.e.*, independent of *de novo* protein synthesis), or if translation of an interferon-induced product is required for the transcription of HERC5. As the expression of secondary (and subsequent) temporal class genes is dependent on the translation of primary temporal class proteins, HERC5 gene-expression during a cycloheximide-maintained translation-block should only be seen if HERC5 is a primary interferon-stimulated gene. To evaluate this, HEL cells were treated with 100 μg/mL of cycloheximide for 30 minutes prior to stimulation with interferon α, in order to effect arrest of translation, and subsequently stimulated with 1000 IU/mL of interferon α. Cycloheximide was maintained in the media of all treated cells to ensure a complete block of translation throughout the experiment. Total RNA was harvested at the indicated time points, and expression of HERC5 was compared to that of ISG 56k, a primary interferon response gene, by Northern blot analysis (20 μg RNA/lane, verified by ethidium bromide staining of ribosomal RNA) (Figure 3.2.3 A). To confirm that the cycloheximide treatment was effective in arresting cellular translation, HSV1 strain KOS1.1-infected HEL cells were analyzed for the induction of a primary temporal gene (ICP0), and a secondary temporal gene (thymidine kinase, TK) when cultured in the presence of cycloheximide (Figure 3.2.3 B). As demonstrated in figure 3.2.2, HERC5, ISG 56k, and ICP0 transcription occurs despite the arrest of translation due to cycloheximide, while the expression of thymidine kinase, a secondary temporal class gene, is reduced to undetectable levels. As such, HERC5 is a member of the primary temporal class of interferon-stimulated genes.

3.2.3. Human HERC5 is Strongly Induced by Poly-Inosinic: Poly-Cytidylic Double-Stranded Ribonucleic Acid but not Interferon γ

Some interferon α-stimulated genes may also be induced in response to other stimuli, such as interferon γ or some Toll-like receptor ligands. To examine the ability of other antiviral stimuli to activate HERC5 transcription, HEL fibroblasts were treated with either 1000 IU/mL of interferon α, 1000 IU/mL of interferon γ, or 25 μg/mL poly-inosinic: poly-cytidylic double-stranded RNA (Poly I:C RNA, a Toll-like receptor 3 ligand). Total mRNA was then harvested at various times post-stimulation, and expression of HERC5 was compared to that of the interferon-stimulated gene ISG 56k by Northern blot analysis. Figure 3.2.3 illustrates that Poly I:C RNA, in addition to interferon α, induced both ISG 56k and HERC5 gene expression, while interferon γ-stimulation leads to detectible levels of ISG 56k only. It is of interest to note that, while interferon α-stimulation of HEL fibroblasts results in a very rapid induction of both ISG 56k and HERC5 expression, the levels of both ISG 56k and HERC5 mRNA are obviously diminished by 12 hours post-stimulation. Stimulation of HEL fibroblasts with Poly I:C RNA, in contrast, displays different kinetics for both transcripts. Treatment with Poly I:C RNA results in a much greater magnitude of HERC5 transcription (and/or stability of HERC5 mRNA) as compared to interferon α treatment, albeit the maximal levels of HERC5 mRNA occur much later after stimulation. Treatment of HEL fibroblasts with Poly I:C RNA also results in a delayed activation of ISG 56k, though maximal transcript levels are comparable to those seen during stimulation with interferon α. These findings suggest that HERC5 is inducible via stimulation of cells with either interferon α or Poly I:C RNA, while interferon γ does not lead to a detectible increase in HERC5 expression. Additionally, the greatly increased levels of HERC5 mRNA seen during stimulation of HEL cells with Poly I:C RNA suggest that maximal activation of HERC5 expression (transcription and/or stability of HERC5 mRNA) may depend on factors exclusive of the interferon α-induced pathway.

3.3. Transient Transfection and Expression of HERC5

3.3.1. HERC5 Protein is Localized to the Cytoplasm

Previous studies have described cell-type specific variations in HERC5 protein localization [143, 253]. The localization of HERC5 has been described as punctate and cytoplasmic in HeLa cells transiently-transfected to express a FLAG-epitope tagged version of HERC5 [143], diffuse cytoplasmic staining in transiently-transfected SaOS-2 cells (untagged HERC5) [253], and perinuclear localization of endogenous HERC5 in SV40-transformed WI-38 human diploid embryonic fibroblasts [253]. To determine if HERC5 localization is cell type-dependent, A549, HEK293T, and U-2 OS cells were transiently-transfected with pGQ06 and analyzed for HERC5 localization by immunofluorescent staining of the FLAG epitope (Figure 3.3.1). In the cell lines examined, FLAG-HERC5 was completely excluded from the nucleus and diffusely distributed throughout the cytoplasm, with occasional punctate bodies. Of great interest, pGQ06-transfected U-2 OS cells frequently demonstrated aberrant nuclear morphologies, while such phenotypic alterations were noted in neither A549 nor HEK293T cells. The nuclear dysmorphisms observed were characterized by abnormal staining patterns of nuclear DNA with DAPI and visible changes in the nuclear structure apparent in the DIC image.

3.3.2. HERC5 Induces in Nuclear Dysmorphisms in U-2 OS Cells

To investigate whether the changes in nuclear morphology seen in U-2 OS cells were associated with HERC5 expression, transfection alone, or the immunofluorescence protocol used, U-2 OS cells were transfected with either pGQ06 (an expression vector for FLAG-HERC5), pGQ13 (an expression vector for human cyclin E), p3xFLAG-CMV10-Dectin 2 (a control expression vector for a FLAG-tagged protein), or pEGFP-C1 (an EGFP expression vector).. All transfected cells were fixed as described in section 2.7. Cells transfected with pGQ06 or p3xFLAG-CMV10-Dectin 2 were stained with rabbit polyclonal anti-FLAG antibodies, while pGQ13 cells were stained with mouse monoclonal anti-cyclin E antibodies. pEGFP-C1-transfected cells were not immunostained, but otherwise treated identically to other transfected cells. Cells were subsequently analyzed by confocal microscopy. As shown in figure 3.3.2 A, cells transfected with pGQ13, p3xFLAG-CMV10-Dectin 2, and pEGFP-C1 consistently demonstrated normal nuclear morphology, suggesting that neither the process of immunostaining nor the transfection of U-2 OS cells were causal factors in the nuclear dysmorphisms seen. As such, the nuclear dysmorphisms seen in U-2 OS cells appears to be unique to transfection with pGQ06, as only pGQ06-transfected cells demonstrated a significant range of aberrant nuclear phenotypes (Figure 3.3.2 B, Table 3.3.2). The efficiency at which transfection of U-2 OS cells with pGQ06 caused nuclear dysmorphisms, and the severity of nuclear distortion, appears to be co-dependent on as-yet unidentified factors as the extent to which nuclear malformations (*e.g.*, bending/stretching of the nucleus, herniation/lobulation, or formation of micronuclei) varied between experiments (Table 3.3.2). Additionally, non-transfected cells with nuclear dysmorphic phenotypes appear slightly more frequently in cultures with pGQ06-transfected cells (Table 3.3.2). The increased occurrence of nuclear dysmorphic cells in pGQ06-transfected cultures may be biased by the inclusion of cells expressing HERC5 below the limits of detection. Alternatively, the increase in nuclear dysmorphic cells may be an effect resulting from the release cytokines or other factors from cells over-expressing HERC5.

3.3.3. HERC5-Induced Nuclear Dysmorphisms in U-2 OS Cells Do Not Correlate with Fragmentation of Nuclear DNA

In response to cellular stresses, such as recognition of irreparable damage to nuclear DNA or deregulation of cell cycle proteins, cells may activate biochemical pathways leading to the death of the cell, such as programmed-like cell death or apoptosis [92, 281]. To determine whether the nuclear dysmorphisms seen in some cells transfected with pGQ06 were related to the activation of such pathways, cells were examined for a characteristic marker of apoptosis: fragmentation of nuclear DNA. To these ends, U-2 OS cells were grown on coverslips, transfected with pGQ06, and incubated for 19 hours to allow expression of FLAG-HERC5. Subsequently, one coverslip of pGQ06-transfected cells was subjected to 500 μJ/cm^2 of ultraviolet irradiation. Cells were further incubated for seven hours. Cells were then analyzed for the expression of FLAG-HERC5, and fragmentation of nuclear DNA by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) (Figure 3.3.3). All ultraviolet-irradiated transfected cells were strongly positive for TUNEL, though no FLAG-HERC5 expression was noted (which may be a product of caspase activation or other mechanisms of enhanced protein degradation or simply cessation of HERC5 transcription and consequent translation). In contrast, non-irradiated pGQ06-transfected cells, even cells demonstrating severe malformations of nuclear structure, were completely negative for TUNEL staining. Thus, the expression of FLAG-HERC5 and nuclear dysmorphisms stemming there from did not correlate with fragmentation of nuclear DNA.

3.3.4. Domains of HERC5 Required for Nuclear Dysmorphisms

To evaluate which regions of HERC5 are necessary for the nuclear structural changes apparent in U-2 OS cells, a series of HERC5 expression vectors was created which included mutants with truncations, deletions, and/or fusions to the HERC5 open reading frame. Various HERC5-expression vectors were transfected into U-2 OS cells, followed by analysis of HERC5 expression and the incidence of nuclear abnormalities by immunofluorescence assay (Figure 3.3.4, Table 3.3.4).

Similar to pGQ06-transfected cells, nuclear malformations are evident in many cells transfected with pGQ14, pGQ15, and pGQ16 (Figure 3.3.4 B-D). pGQ14 and pGQ15 encode fusions of HERC5 to the amino terminus of EGFP, with an 11-amino acid truncation to the carboxy-terminus of HERC5. As HECT domains typically do not function unless located at the extreme carboxy terminus of a protein [101, 155, 184, 337], and HECT function is currently thought to require a "-4" phenylalanine residue [337] (which is deleted in both pGQ14 as well as pGQ15), neither pGQ14 nor pGQ15 should demonstrate the ability to function as a ubiquitin E3 ligases, though they should retain the ability to form ubiquitin thioesters via the catalytic cysteine (C994) [337]. While pGQ16 contains only a single amino acid substitution (C994A) it should not be able form the ubiquitin thioester intermediate due to mutation of a predicted catalytic cysteine residue, and thus also expected lack ubiquitin E3 ligase function [347]. As abrogation of HECT domain function appears to have little effect on the extent to which nuclear abnormalities occurred, the data suggest that HECT domain function is not required for the nuclear malformations seen (Figure 3.3.4 B-D, Table 3.3.4). Additionally, the complete colocalization of FLAG epitope staining and the enhanced-GFP domain following transfection with pGQ15 strongly suggest that these fusions are not proteolytically separated and that detection of such epitopes or domains accurately reflects the localization of the HERC5 variants as intended.

The HERC5-expression vector pGQ18 is a variant of pGQ14 lacking the first 347 amino acids (which encodes all but 30 amino acids of the RCC1 domain). As such, the protein product of pGQ18 would be expected to lack functional RCC1 and HECT domains, but may still retain protein interactions derived from the last 30 amino acids of the RCC1 domain. When transfected into U-2 OS cells, pGQ18 results in a similar incidence of nuclear abnormalities as seen with pGQ06 (Figure 3.3.4 B,C, Table 3.3.4), thus a functional RCC1 domain is not required for the nuclear effects seen.

pGQ22 is a HERC5-expression vector encoding a triple FLAG epitope tag fused to a HERC5 variant bearing a 444 amino acid amino-terminal truncation (completely deleting the RCC1 domain and a further 66 amino acids of the putative cyclin-binding region). Thus, the protein product of pGQ22 would be expected to completely lack RCC1 function, which retaining HECT domain function. Due to the lack of knowledge of the putative cyclin-binding region, it is not apparent what effect the deletion would have on this region of the protein. Figure 3.3.4 B illustrates that while transfection of pGQ22 still resulted in nuclear abnormalities in a large proportion of transfected cells, these nuclear abnormalities occurred in a significantly lower proportion of transfected cells (Table 3.3.4). This result suggests that neither the function of the RCC1 nor the HECT domain of HERC5 is essential for the occurrence nuclear dysmorphisms seen in U-2 OS cells, but rather other functions or protein interactions (which may be partially compromised through the HERC5 deletions encoded in pGQ22) may be necessary for the occurrence of this effect.

3.3.5. Localization of Cyclins in Response to HERC5 Expression

HERC5 appears to be exclusively localized to the cytoplasm, while most cyclins are thought to be chiefly active in the nucleus. As HERC5 is proposed to have cell cycle modulatory functions [250, 253], it may function by sequestering cyclins in the cytoplasm and preventing their nuclear import. To determine if

HERC5 expression leads to appreciable changes in cyclin localization, pGQ06 was transfected into U-2 OS cells using FuGENE6. The localization of ectopically-expressed FLAG-HERC5 and endogenously-expressed cyclins A, B, and D were identified by confocal microscopy following immunofluorescent staining of cells (Figure 3.3.5 A).

Immunostaining U-2 OS cells for endogenous cyclin A demonstrated a significant population of cells with nuclear staining, while all cells bore little to no cytoplasmic staining. In cells with strong cyclin A staining and expressing FLAG-HERC5, cyclin A localization does not appear to be altered.

Cyclin B immunostaining of U-2 OS cells demonstrates that cyclin B is primarily cytoplasmic and present in nearly all cells. Strong staining was rarely seen, but in such cells increased nuclear and perinuclear localization was noted. Cells with strong cyclin B staining and expression of FLAG-HERC5 were not found, though expression of FLAG-HERC5 had no detectable effect in cells expressing low levels of cyclin B.

Strong (near-exclusively) nuclear staining of cyclin D, using both anti-cyclin D1 and anti-cyclin D3 antibodies, was seen in every cell, irregardless of FLAG-HERC5 expression. Expression of FLAG-HERC5 did not appear to alter the localization of cyclin D.

Virtually all non-transfected U-2 OS cells demonstrated strong nuclear staining with anti-cyclin E (Figure 3.3.5 B). While little to no cytoplasmic anti-cyclin E staining was noted in cells not expressing FLAG-HERC5, cells expressing FLAG-HERC5 frequently demonstrated lesser nuclear staining and greater cytoplasmic staining of cyclin E, suggesting that FLAG-HERC5 may alter the localization of cyclin E.

To control for non-specific effects do to transfection or ectopic protein expression, pEGFP-C1 was used as a control vector. As was noted when pGQ06 was transfected into U-2 OS cells, nearly all cells demonstrated strong nuclear staining of cyclin E. However, several cells transfected with pEGFP-C1 demonstrated strong cytoplasmic and low nuclear staining of cyclin E, suggesting that cyclin E-localization differences seen with FLAG-HERC5 may be non-specific and rather an artefact of the transfection procedure (Figure 3.3.5 B).

To further examine the possibility that HERC5 expression may affect cyclin E localization, co-transfection experiments using vectors encoding cyclin E and FLAG-HERC5 (pGQ13 and pGQ06, respectively) were performed. When pGQ13 was transfected into U-2 OS cells alone, cyclin E was strongly expressed and demonstrated near-complete nuclear localization in most cells (Figure 3.3.5 C). However, when pGQ13 was co-transfected with pGQ06 a significant increase in the cytoplasmic retention of cyclin E was observed, though strong nuclear staining was typically still present. As such, this suggests that HERC5 and cyclin E may interact in the cytoplasm. However, as nuclear accumulation of cyclin E continued despite the over-expression of HERC5, it seems HERC5 does not effectively block cyclin nuclear import.

3.4. Stable Transfection of and Inducible Expression of HERC5

3.4.1. HERC5 Expression Does Not Affect Cyclin A Levels

To determine if expression of HERC5 affects levels of cyclins, potentially through the action of the HECT ubiquitin ligase domain, cell lines capable of tetracycline-repressed expression of FLAG-HERC5 were created from UTA6 cells transfected with pGQ08. Cells were plated in replicate cultures, with FLAG-HERC5 repression maintained by doxycycline in one plate of each replicate culture. In the other culture, selection medium free of tetracyclines was utilized. Eighteen hours after plating, cells were rinsed twice with PBS, and media replaced with selection medium with or without doxycycline (lacking puromycin for non-transfected UTA6). Cells were grown for a further 30 hours, and then lysed in SDS-PAGE lysis buffer. Samples were boiled at 95°C for 5 minutes, followed by resolution of samples on 4%, 10% discontinuous SDS-PAGE gels. Cyclin A was chosen for study due to specificity of the antibody in western blotting, and the relatively high level of endogenous cyclin A expression in U-2 OS cells (Figure 3.3.5 A). Levels of cyclin A were qualitatively analysed by western blotting and compared between uninduced and cells induced to express

FLAG-HERC5 protein. Figure 3.4.1 illustrates no significant change in cyclin A signal intensity, nor were any additional band products (such as ubiquitinated cyclin A or other covalent modifications) noted. Thus, under the conditions of this experiment, FLAG-HERC5 does not appear to significantly affect cellular levels of cyclin A, nor affect its migration on an SDS-PAGE gel, suggesting that HERC5 does not function to covalently modify cyclins.

Figure 3.1.1: Multiple Alignment of HERC5 cDNA Primary Structure. The "NCBI Genome" and "Celera Genome" predicted cDNA sequences were generated from the NCBI Reference and Celera human genome sequences, respectively, using bioinformatic mRNA splice-modeling software (Model Maker, www.ncbi.nlm.nih.gov). The reported cDNA sequences AB027289 [2] and AY337518 [1] have been previously described. The cDNA sequence of pGQ01 was generated through sequencing the open reading frame of the plasmid. All sequences have been aligned and truncated to begin at the HERC5 initiation codon and end at the HERC5 termination codon. Identities to the NCBI Reference sequence are noted with a ".", while deviations are indicated by the substituted nucleotide.

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                          10         20         30         40         50         60         70         80         90        100
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome       ATGGAGCGGA GGTCGCGGAG GAAGTCGCGG CGCAACGGGC GCTCGACCGC GGGCAAGGCC GCCGCGACCC AGCCCGCGAA GTCTCCGGGC GCACAGCTCT
Celera Genome     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                         110        120        130        140        150        160        170        180        190        200
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome       GGCTCTTTCC CAGCGCCGCG GGCCTCCACC GCGCGCTGCT CCGGAGGGTG GAGGTGACGC GCCAACTCTG CTGCTCGCCG GGGCGCCTCG CGGTCTTGGA
Celera Genome     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                         210        220        230        240        250        260        270        280        290        300
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome       ACGCGGCGGG GCGGGCGTCC AGGTTCACCA GCTGCTCGCC GGGAGCGGCG GCGCCCGGAC GCCGAAATGC ATTAAATTAG GAAAAAACAT GAAGATACAT
Celera Genome     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                         310        320        330        340        350        360        370        380        390        400
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome       TCCGTGGACC AAGGAGCAGA GCACATGCTG ATTCTCTCAT CAGATGGAAA ACCATTTGAG TATGACAACT ATAGCATGAA ACATCTAAGG TTTGAAAGCA
Celera Genome     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                         410        420        430        440        450        460        470        480        490        500
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome       TTTTACAAGA AAAAAAAATA ATTCAGATCA CATGTGGAGA TTACCATTCT CTTGCACTCT CAAAAGGTGG TGAGCTTTTT GCCTGGGGAC AGAACCTGCA
Celera Genome     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289          .......... .......... .......... .......... .......... .......... .......C.. .......... .......... ..........
AY337518          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01             .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                     510        520        530        540        550        560        570        580        590        600
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome   TGGGCAGCTT GGAGTTGGAA GGAAATTTCC CTCAACCACC ACACCACAGA TTGTGGAGCA CCTCGCAGGA GTACCCTTGG CTCAGATTTC TGCCGGAGAA
Celera Genome .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     610        620        630        640        650        660        670        680        690        700
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome   GCCCACAGCA TGGCCTTATC CATGTCTGGC AACATTTATT CATGGGGAAA AAATGAATGT GGACAACTAG GCCTGGGCCA CACTGAGAGT AAAGATGATC
Celera Genome .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518      .......... C......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     710        720        730        740        750        760        770        780        790        800
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome   CATCCCTTAT TGAAGGACTA GACAATCAGA AAGTTGAATT TGTCGCTTGT GGTGGCTCTC ACAGTGCCCT ACTCACACAG GATGGGCTGC TGTTTACTTT
Celera Genome .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     810        820        830        840        850        860        870        880        890        900
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome   CGGTGCTGGA AAACATGGGC AACTTGGTCA TAATTCAACA CAGAATGAGC TAAGACCCTG TTTGGTGGCT GAGCTTGTTG GGTATAGAGT GACTCAGATA
Celera Genome .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     910        920        930        940        950        960        970        980        990       1000
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome   GCATGTGGAA GGTGGCACAC ACTTGCCTAT GTTTCTGATT TGGGAAAGGT CTTTTCCTTT GGTTCTGGAA AAGATGGACA ACTGGGAAAT GGTGGAACAC
Celera Genome .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                     1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     GTGACCAGCT GATGCCGCTT CCAGTGAAAG TATCATCAAG TGAAGAACTC AAACTTGAAA GCCATACCTC AGAAAAGGAG TTAATAATGA TTGCTGGAGG
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     GAATCAAAGC ATTTTGCTCT GGATAAAGAA AGAGAATTCA TATGTTAATC TGAAGAGGAC AATTCCTACT CTGAATGAAG GGACTGTAAA GAGATGGATT
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     GCTGATGTGG AGACTAAACG GTGGCAGAGC ACAAAAAGGG AAATCCAAGA GATATTTTCA TCTCCTGCTT GTCTAACTGG AAGTTTTTTA AGGAAAAGAA
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... ....C..... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     GAACTACAGA AATGATGCCT GTTTATTTGG ACTTAAATAA AGCAAGAAAC ATCTTCAAGG AGTTAACCCA AAAGGACTGG ATTACTAACA TGATAACCAC
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......G.. .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     CTGCCTCAAA GATAATCTGC TCAAAAGACT TCCATTTCAT TCTCCACCCC AAGAAGCTTT AGAAATTTTC TTCCTTCTCC CAGAATGTCC TATGATGCAT
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... .G........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... .G........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... .G........
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                     1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    ATTTCCAACA ACTGGGAGAG CCTTGTGGTT CCATTTGCAA AGGTTGTTTG TAAAATGAGT GACCAGTCTT CACTGGTTCT GGAAGAGTAT TGGGCAACTC
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... ......A... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TGCAAGAATC CACTTTCAGC AAACTGGTCC AGATGTTTAA AACAGCCGTC ATATGCCAGT TGGATTACTG GGATGAAAGT GCTGAGGAGA ATGGTAATGT
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TCAAGCTCTC CTAGAAATGT TGAAGAAGCT GCACAGGGTA AACCAGGTGA AATGTCAACT ACCTGAAAGT ATTTTCCAAG TAGACGAACT CTTGCACCGT
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    CTCAATTTTT TTGTAGAAGT ATGCAGAAGG TACTTGTGGA AAATGACTGT GGACGCTTCA GAAAATGTAC AATGCTGCGT CATATTCAGT CACTTTCCAT
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1910       1920       1930       1940       1950       1960       1970       1980       1990       2000
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TTATCTTTAA TAATCTGTCG AAAATTAAAC TACTACATAC AGACACACTT TTAAAAATAG AGAGTAAAAA ACATAAAGCT TATCTTAGGT CGGCAGCAAT
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                      2010       2020       2030       2040       2050       2060       2070       2080       2090       2100
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TGAGGAAGAA AGAGAGTCTG AATTCGCTTT GAGGCCCACG TTTGATCTAA CAGTCAGAAG GAATCACTTG ATTGAGGATG TTTTGAATCA GCTAAGTCAA
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      2110       2120       2130       2140       2150       2160       2170       2180       2190       2200
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TTTGAGAATG AAGACCTGAG GAAAGAGTTA TGGGTTTCAT TTAGTGGAGA AATTGGGTAT GACCTCGGAG GAGTCAAGAA AGAGTTCTTC TACTGTCTGT
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      2210       2220       2230       2240       2250       2260       2270       2280       2290       2300
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TTGCAGAGAT GATCCAGCCG GAATATGGGA TGTTCATGTA TCCTGAAGGG GCTTCCTGCA TGTGGTTTCC TGTCAAGCCT AAATTTGAGA AGAAAAGATA
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      2310       2320       2330       2340       2350       2360       2370       2380       2390       2400
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    CTTCTTTTTT GGGGTTCTAT GTGGACTTTC CCTGTTCAAT TGCAATGTTG CCAACCTTCC TTTCCCACTG GCACTGTTTA AGAAACTTTT GGACCAAATG
Celera Genome  .......... .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......C.. .......... .......... .......... ..........

                      2410       2420       2430       2440       2450       2460       2470       2480       2490       2500
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    CCATCATTGG AAGACTTGAA AGAACTCAGT CCTGATTTGG GAAAGAATTT GCAAACACTT CTGGATGATG AAGGTGATAA CTTTGAGGAA GTATTTTACA
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                      2510       2520       2530       2540       2550       2560       2570       2580       2590       2600
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     TCCATTTTAA TGTGCACTGG GACAGAAACG ACACAAACTT AATTCCTAAT GGAAGTAGCA TAACTGTCAA CCAGACTAAC AAGAGAGACT ATGTTTCTAA
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      2610       2620       2630       2640       2650       2660       2670       2680       2690       2700
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     GTATATCAAT TACATTTTCA ACGACTCTGT AAAGGCGGTT TATGAAGAAT TTCGGAGAGG ATTTTATAAA ATGTGCGACG AAGACATTAT CAAATTATTC
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      2710       2720       2730       2740       2750       2760       2770       2780       2790       2800
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     CACCCCGAAG AACTGAAGGA TGTGATTGTT GGAAATACAG ATTATGATTG GAAAACATTT GAAAAGAATG CACGTTATGA ACCAGGATAT AACAGTTCAC
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      2810       2820       2830       2840       2850       2860       2870       2880       2890       2900
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     ATCCCACCAT AGTGATGTTT TGGAAGGCTT TCCACAAATT GACTCTGGAA GAAAAGAAAA AATTCCTTGT ATTTCTTACA GGAACTGACA GACTACAAAT
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      2910       2920       2930       2940       2950       2960       2970       2980       2990       3000
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome     GAAAGATTTA AATAATATGA AAATAACATT TTGCTGTCCT GAAAGTTGGA ATGAAAGAGA CCCTATAAGA GCACTGACAT GTTTCAGTGT CCTCTTCCTC
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                      3010       3020       3030       3040       3050       3060       3070
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|
NCBI Genome     GCTAAATATT CTACAATGGA AACAGTTGAA GAAGCGCTTC AAGAAGCCAT CAACAACAAC AGAGGATTTG GCTGA
Celera Genome   .......... .......... .......... .......... .......... .......... .......... .....
AB027289        .......... .......... .......... .......... .......... .......... .......... .....
AY337518        .......... .......... .......... .......... .......... .......... .......... .....
pGQ01           .......... .......... .......... .......... .......... .......... .......... .....
```

Figure 3.1.2: The 3' Untranslated Region of Human HERC5 mRNA Contains a Repeated GTT Motif.

The "NCBI Genome" and "Celera Genome" cDNA representations of HERC5 mRNA were generated from the NCBI Reference and Celera human genome sequences, respectively, using bioinformatic mRNA splice-modeling software (Model Maker, www.ncbi.nlm.nih.gov). The reported cDNA sequences AB027289 [2] and AY337518 [1] have been previously described. The cDNA sequences of pGQ01 and pGQ03 were generated by the sequencing of those plasmids. All sequences have been aligned and truncated to begin at the HERC5 termination codon. Twelve repeats of the GTT motif occur, from the 33rd to 90th nucleotides of the alignment, in the cDNA representations derived from the NCBI reference and Celera genomic sequences, the experimentally-obtained cDNA copies of HERC5 mRNA, and the sequence of pGQ01, but have been removed from the sequence of pGQ03.

```
                        10         20         30         40         50         60         70         80         90        100
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TGACCAGCTT GCTTGTCCAA CAGCCTTATT TTGTTGTTGT TATCGTTGTT GTTGTTGTTG TTGTTGTTGT TTCTCTACTT TGTTTTGTTT TAGGCTTTTA
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ03          .......... ...C.AG

                       110        120        130        140        150        160        170        180        190        200
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    GCAGCCTGAA GCCATGGTTT TTCATTTCTG TCTCTAGTGA TAAGCAGGAA AGAGGGATGA AGAAGAGGGT TTACTGGCCG GTTAGAACCC GTGACTGTAT
Celera Genome  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB027289       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ03

                       210        220        230        240        250        260        270        280        290        300
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NCBI Genome    TCTCTCCCTT GGATACCCCT ATGCCTACAT CATATTCCTT ACCTCTTTTG GGAAATATTT TTCAAAAATA AAATAACCAA AAATTAACAT AA
Celera Genome  .......... .......... .......... .......... .......... .......... .......... ........G. ...A.T.ACA T.A
AB027289       .......... .......... .......... .......... .......... .......... .......... ........G. ...ACT.A.A ..AGAAAAAA
AY337518       .......... .......... .......... .......... .......... .......... .......... ........G. .......A.A ..AAAAAAAA
pGQ01          .......... .......... .......... .......... .......... .......... .........G GCC.TCGTGG CCT.CGTGGC CTCGAG
pGQ03
```

Figure 3.1.3: Comparison of the Predicted Protein Products of Various HERC5 cDNA Sequences.

Based on the predicted primary structure of HERC5 mRNA, as derived from the NCBI Reference and Celera human genome sequences, the predicted primary structure of human HERC5 protein was derived. These sequences were then compared with those predicted by translation of the experimentally-derived cDNA sequences of HERC5 mRNA (AB027289 [2] and AY337518 [1]), as well as that predicted from the open reading frame of pGQ01. The location of the C994A point mutation encoded within pGQ16 is also illustrated.

```
                                        10         20         30         40         50         60         70         80         90        100
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence MERRSRRKSR RN RSTA KA AATQPAKSP  AQLWLFPSAA  LHRALLRRV EVTRQLCCSP  RLAVLER   A VQVHQLLA  S  ARTPKC IKL KNMKIH
AB027289 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                       110        120        130        140        150        160        170        180        190        200
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence SVDQ AEHML ILSSD KPFE YDNYSMKHLR FESILQEKKI IQITC DYHS LALSK  ELF AW QNLH QL  V RKFPSTT TPQIVEHLA  VPLAQISA E
AB027289 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                       210        220        230        240        250        260        270        280        290        300
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence AHSMALSMS  NIYSW KNEC  QL L HTES KDDPSLIE L DNQKVEFVAC   SHSALLTQ D LLFTF A  KH QL HNST QNELRPCLVA ELV YRVTQI
AB027289 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated            ...T...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                       310        320        330        340        350        360        370        380        390        400
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence AC RWHTLAY VSDL KVFSF  S KD QL N   TRDQLMPL PVKVSSSEEL KLESHTSEKE LIMIA  NQS ILLWIKKENS YVNLKRTIPT LNE TVKRWI
AB027289 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                       410        420        430        440        450        460        470        480        490        500
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence ADVETKRWQS TKREIQEIFS SPACLT SFL RKRRTTEMMP VYLDLNKARN IFKELTQKDW ITNMITTCLK DNLLKRLPFH SPPQEALEIF FLLPECPMMH
AB027289 Translated            .......... .......... .......... .......... .......... ..R....... .......... .......... .......... .......V..
AY337518 Translated            .......... .........S .......... .......... .......... .......... .......... .......... .......... .......V..
pGQ01 Translated               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......V..

                                       510        520        530        540        550        560        570        580        590        600
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence ISNNWESLVV PFAKVVCKMS DQSSLVLEEY WATLQESTFS KLVQMFKTAV ICQLDYWDES AEEN NVQAL LEMLKKLHRV NQVKCQLPES IFQVDELLHR
AB027289 Translated            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated            .......... ..T....... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated               .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
```

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                                         610        620        630        640        650        660        670        680        690        700
                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence   LNFFVEVCRR YLWKMTVDAS ENVQCCVIFS HFPFIFNNLS KIKLLHTDTL LKIESKKHKA YLRSAAIEEE RESEFALRPT FDLTVRRNHL IEDVLNQLSQ
AB027289 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated                 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                         710        720        730        740        750        760        770        780        790        800
                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence   FENEDLRKEL WVSFS EI Y DL  VKKEFF YCLFAEMIQP EY MFMYPE  ASCMWFPVKP KFEKKRYFFF  VLC LSLFN CNVANLPFPL ALFKKLLDQM
AB027289 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated                 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                         810        820        830        840        850        860        870        880        890        900
                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence   PSLEDLKELS PDL KNLQTL LDDE DNFEE VFYIHFNVHW DRNDTNLIPN  SSITVNQTN KRDYVSKYIN YIFNDSVKAV YEEFRR FYK MCDEDIIKLF
AB027289 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated                 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                                         910        920        930        940        950        960        970        980        990       1000
                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Modelled from Genomic Sequence   HPEELKDVIV  NTDYDWKTF EKNARYEP Y NSSHPTIVMF WKAFHKLTLE EKKKFLVFLT  TDRLQMKDL NNMKITFCCP ESWNERDPIR ALTCFSVLFL
AB027289 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY337518 Translated              .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ01 Translated                 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
pGQ16 cDNA Sequence              .......... .......... .......... .......... .......... .......... .......... .......... .......... ....A.....

                                        1010       1020
                                 ....|....| ....|....| ....
Modelled from Genomic Sequence   PKYSTMETVE EALQEAINNN R F
AB027289 Translated              .......... .......... ....
AY337518 Translated              .......... .......... ....
pGQ01 Translated                 .......... .......... ....
pGQ16 cDNA Sequence              .......... .......... ....
```

Figure 3.2.1: HERC5 is an Interferon-Stimulated Gene.

Total RNA was harvested from human cell lines treated with 1,000 IU/mL human leukocyte-derived interferon α for the indicated periods of time. Each lane contains 20 μg of RNA and was probed for HERC5 and ISG 56k expression by northern blotting. Molecular weight markers are indicated on the left side, and are measured in thousands of base pairs (kBP). The ethidium bromide-stained agarose gel is also shown to demonstrate the equality of RNA loading.

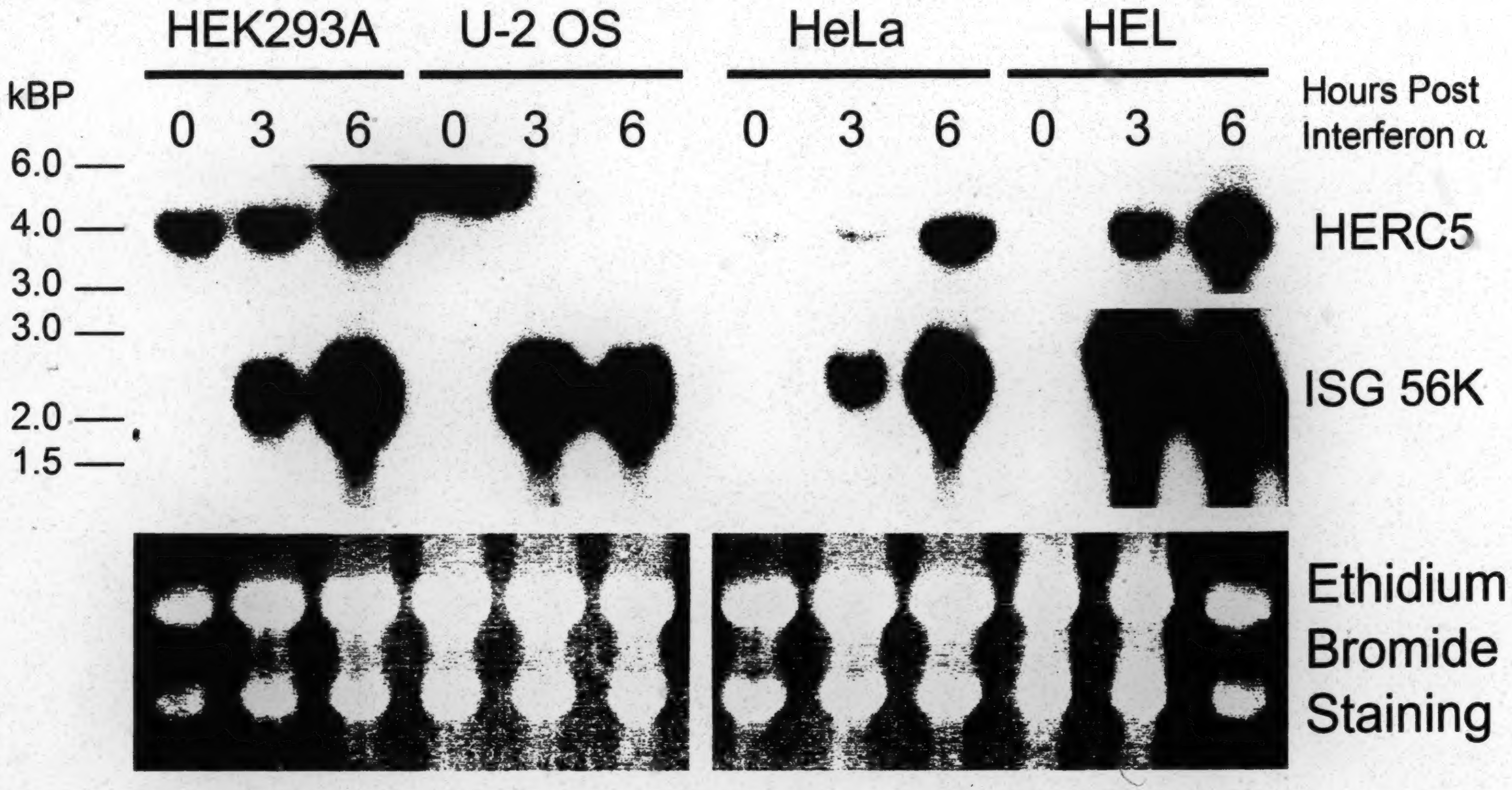
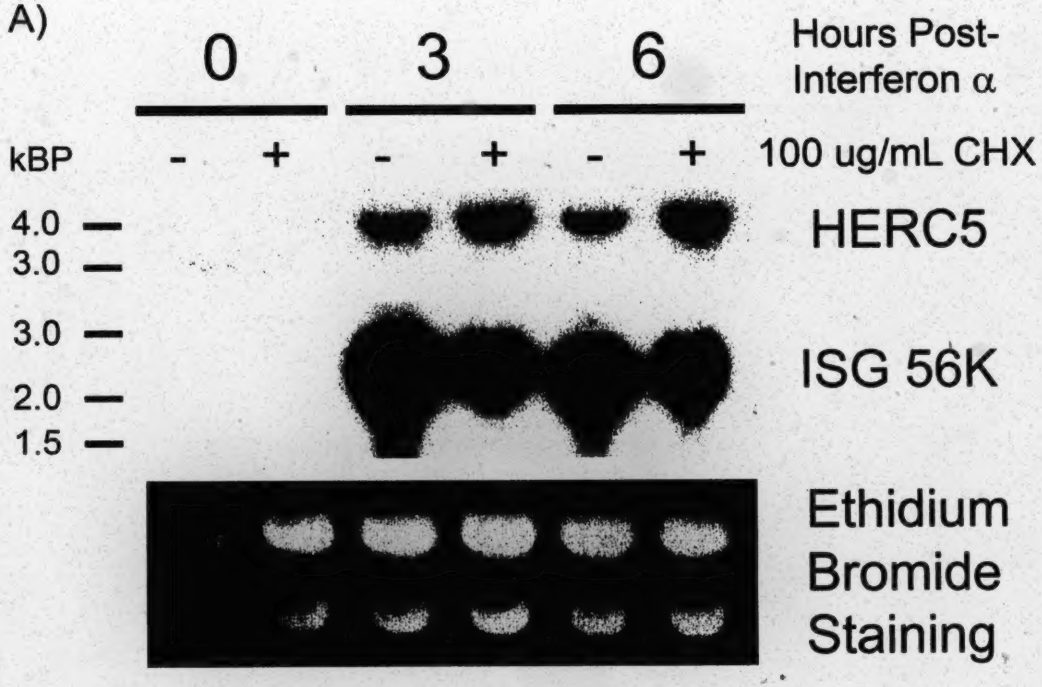

HEK293A
U-2 OS
HeLa
HEL
kBP
0 3 6 0 3 6 0 3 6 0 3 6
Hours Post Interferon α
6.0
4.0
3.0
3.0
2.0
1.5
HERC5
ISG 56K
Ethidium Bromide Staining

Figure 3.2.2: HERC5 is a Primary Interferon-Stimulated Gene.

A) HERC5 transcription in response to stimulation with interferon α is independent of cellular translation. Total RNA was harvested from HEL fibroblasts treated with 1,000 IU/mL human leukocyte-derived interferon α for the indicated periods of time. The pre-treatment and continued culture of cells with 100 μg/mL cycloheximide (CHX) is indicated by "+" above respective lanes. Each lane contains 20 μg of total RNA and was probed for HERC5 and ISG 56k expression by northern blotting. Molecular weight markers are indicated on the left side, and are measured in thousands of base pairs (kBP). The ethidium bromide-stained agarose gel is also shown to demonstrate the equality of RNA loading.

B) The concentration and pre-treatment time of cycloheximide was sufficient to block translation from a strong promoter. HEL fibroblasts were either treated with cycloheximide (CHX), as performed in figure 3.2.2 A, or mock-treated, and subsequently infected cells with ten plaque-forming units of HSV-1 KOS1.1. Total RNA was harvested six hours post-infection. Each lane contains 20 μg of total RNA, and was probed for a primary viral transcript (ICP0) and a secondary viral transcript (viral thymidine kinase) by northern blotting. The ethidium bromide-stained agarose gel is also shown to demonstrate the equality of RNA loading.


A)
0
3
6
Hours Post-
Interferon α
kBP
-
+
-
+
-
+
100 ug/mL CHX
4.0
3.0
HERC5
3.0
2.0
1.5
ISG 56K
Ethidium
Bromide
Staining

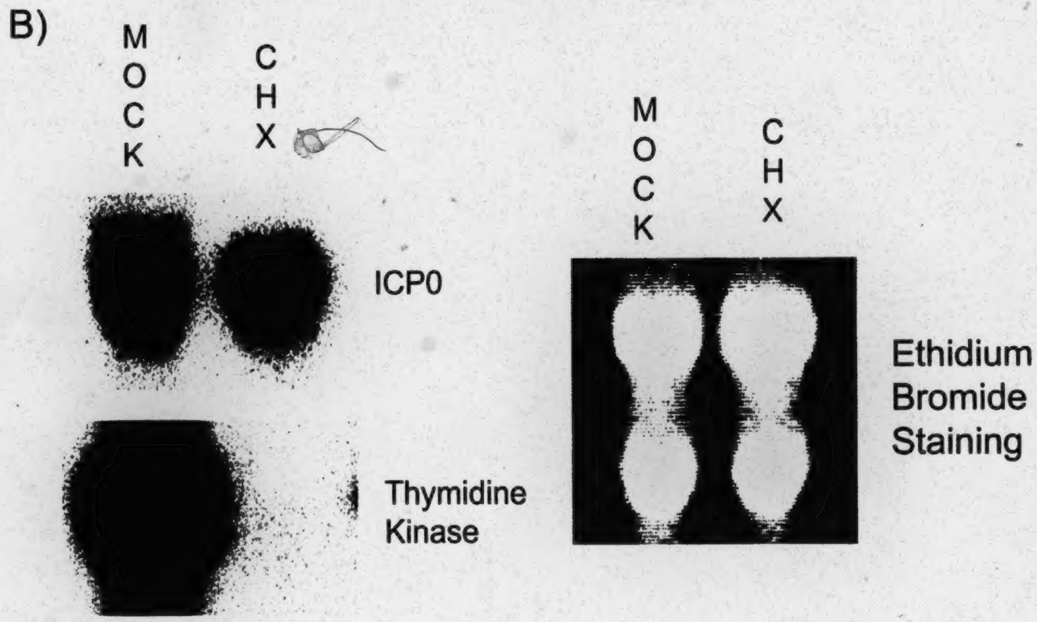

B)
MOCK
CHX
ICP0
Thymidine
Kinase
MOCK
CHX
Ethidium
Bromide
Staining

Figure 3.2.3: HERC5 is Inducible by Both Poly-Inosinic: Cytidylic Ribonucleic Acid and Interferon α, but not Interferon γ.

Total RNA was harvested from HEL cells stimulated with either 1,000 IU/mL human leukocyte-derived interferon α, 1,000 IU/mL recombinant human interferon γ, or 25 μg/mL poly inosinic: cytidylic double-stranded ribonucleic acid for the indicated periods of time. Each lane contains 10 μg of RNA and was probed for HERC5 and ISG 56k expression by northern blotting, as well as for glyceraldehydes-3-phosphate dehydrogenase to demonstrate equal RNA loading. Molecular weight markers are indicated on the left side, and are measured in thousands of base pairs (kBP).

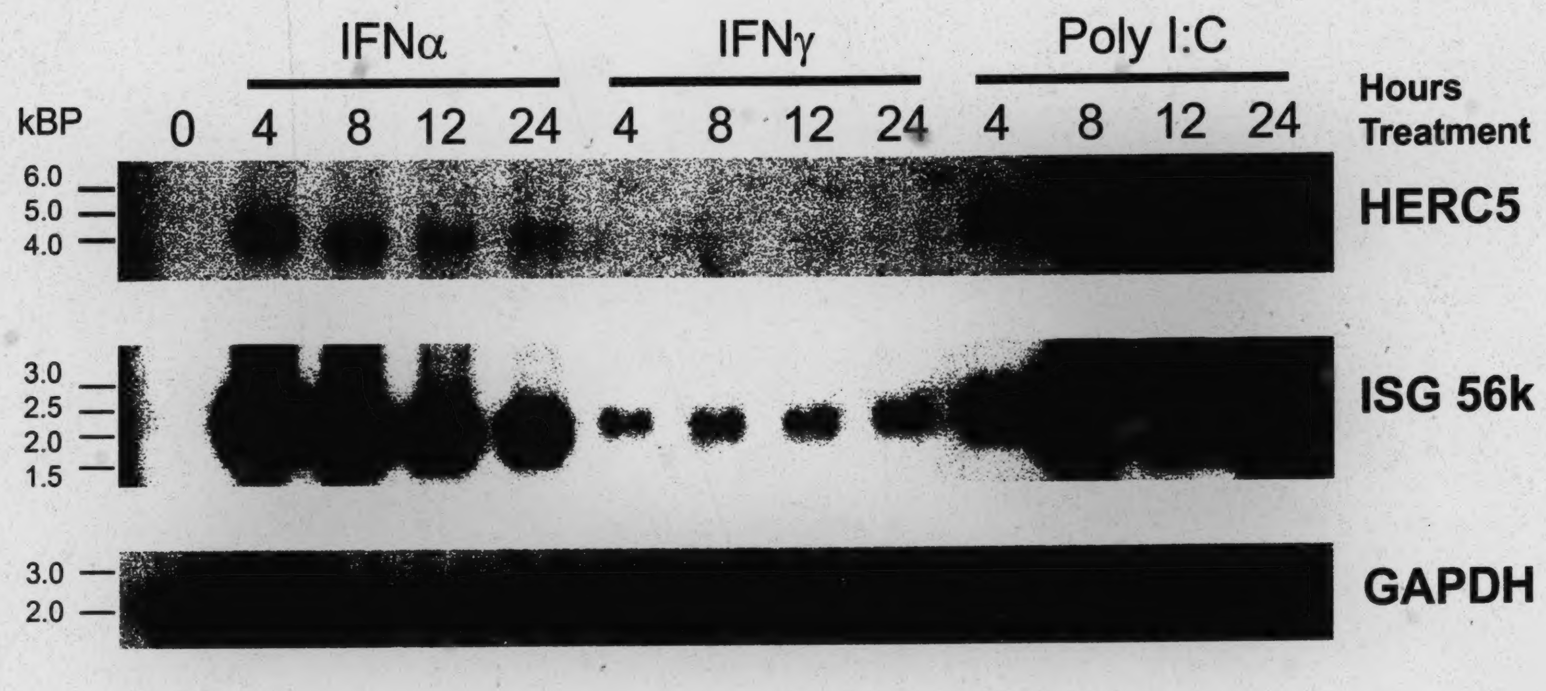

IFNα
IFNγ
Poly I:C
Hours
Treatment
kBP
0
4
8
12
24
4
8
12
24
4
8
12
24
HERC5
ISG 56k
GAPDH
6.0
5.0
4.0
3.0
2.5
2.0
1.5
3.0
2.0

Figure 3.3.1: HERC5 Demonstrates Diffuse Cytoplasmic Localization.

The human cell lines A549, HEK 293T, and U-2 OS were transiently transfected with pGQ06, a mammalian-expression vector encoding HERC5 with an amino-terminal FLAG-epitope tag, using FuGENE6. Eighteen hours post-transfection, cells were fixed, and permeablized. Immunofluorescent localization of ectopically-expressed HERC5 was performed using affinity-purified rabbit polyclonal antibodies to the FLAG epitope and ALEXA 546-conjugated secondary antibodies, with DAPI used as a nuclear counterstain. Differential interference contrast (DIC) images are included for reference. Scale bars in the merged image are 10μm.

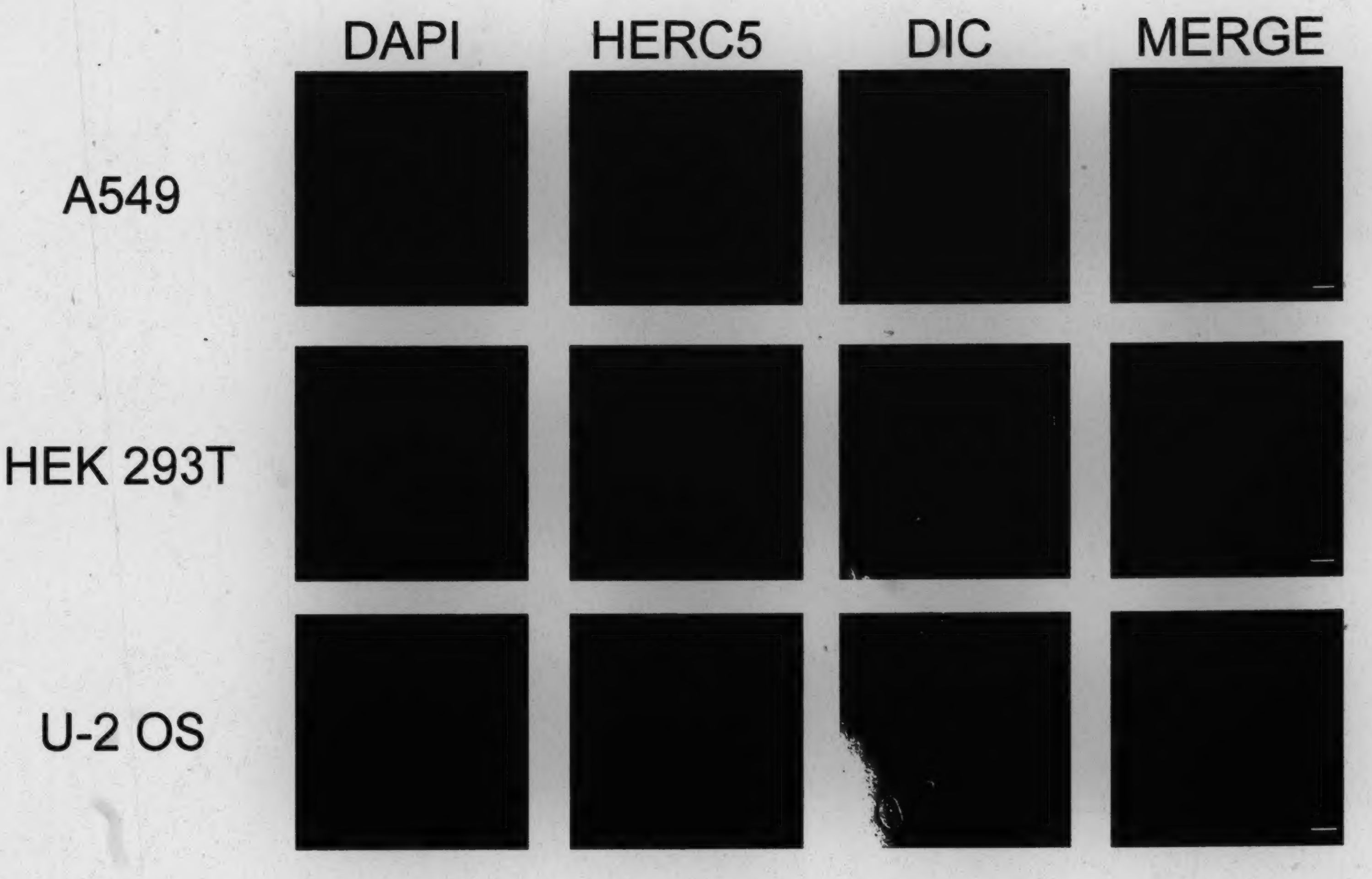
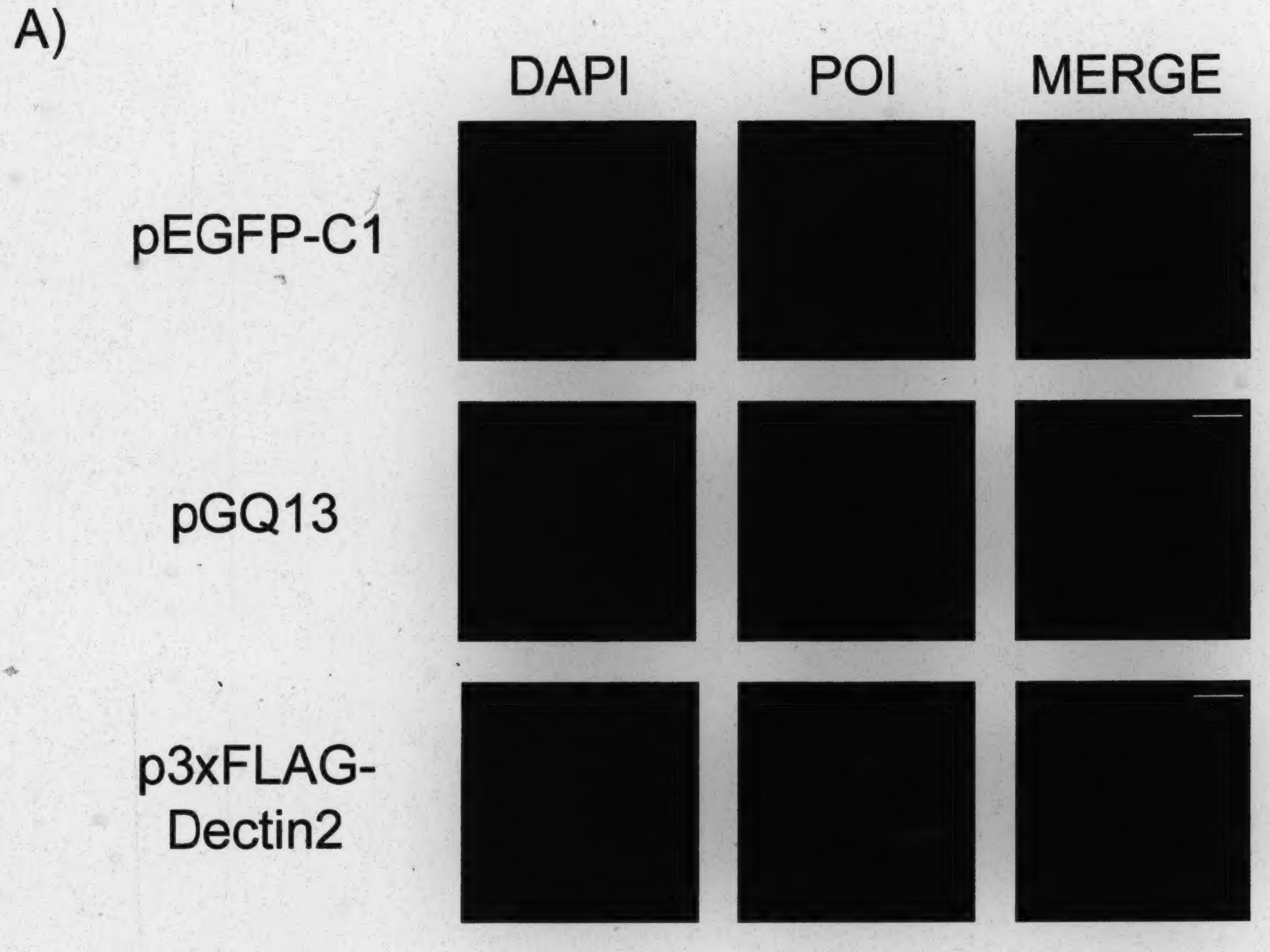

DAPI
HERC5
DIC
MERGE
A549
HEK 293T
U-2 OS

Figure 3.3.2: Ectopic Expression of HERC5 in U-2 OS Cells Induces Abnormal Nuclear Morphologies

A) The nuclear dysmorphisms seen in U-2 OS cells are specific to ectopic HERC5 expression. U-2 OS cells were transfected with either pGQ13, p3xFLAG-CMV10-Dectin 2, or pEGFP-C1 using FuGENE6. Cells were fixed and permeablized as described in section 2.7. POI indicates Protein-of-Interest, as expressed from the vector noted in the left column. Expression of FLAG epitope tagged murine Dectin 2 (p3xFLAG-CMV10-Dectin 2) was detected using rabbit polyclonal anti-FLAG antibodies, while pGQ13 cells were stained with mouse monoclonal anti-cyclin E antibodies. pEGFP-C1-transfected cells were not immunostained, but otherwise treated identically to other transfected cells. Cells were subsequently analyzed by confocal microscopy. Scale bars in the merged image are 10μm.

B) Variation in the nuclear dysmorphic phenotype of U-2 OS cells following expression of HERC5 from pGQ06. U-2 OS cells were transfected with pGQ06 using FuGENE6, and were subsequently fixed and permeablized as described in section 2.7. POI indicates the Protein-of-Interest, HERC5. FLAG epitope tagged HERC5 was detected using rabbit polyclonal anti-FLAG antibodies. Cells were subsequently analyzed by confocal microscopy. Scale bars in the merged image are 10μm.


A)
DAPI
POI
MERGE
pEGFP-C1
pGQ13
p3xFLAG-Dectin2

B)

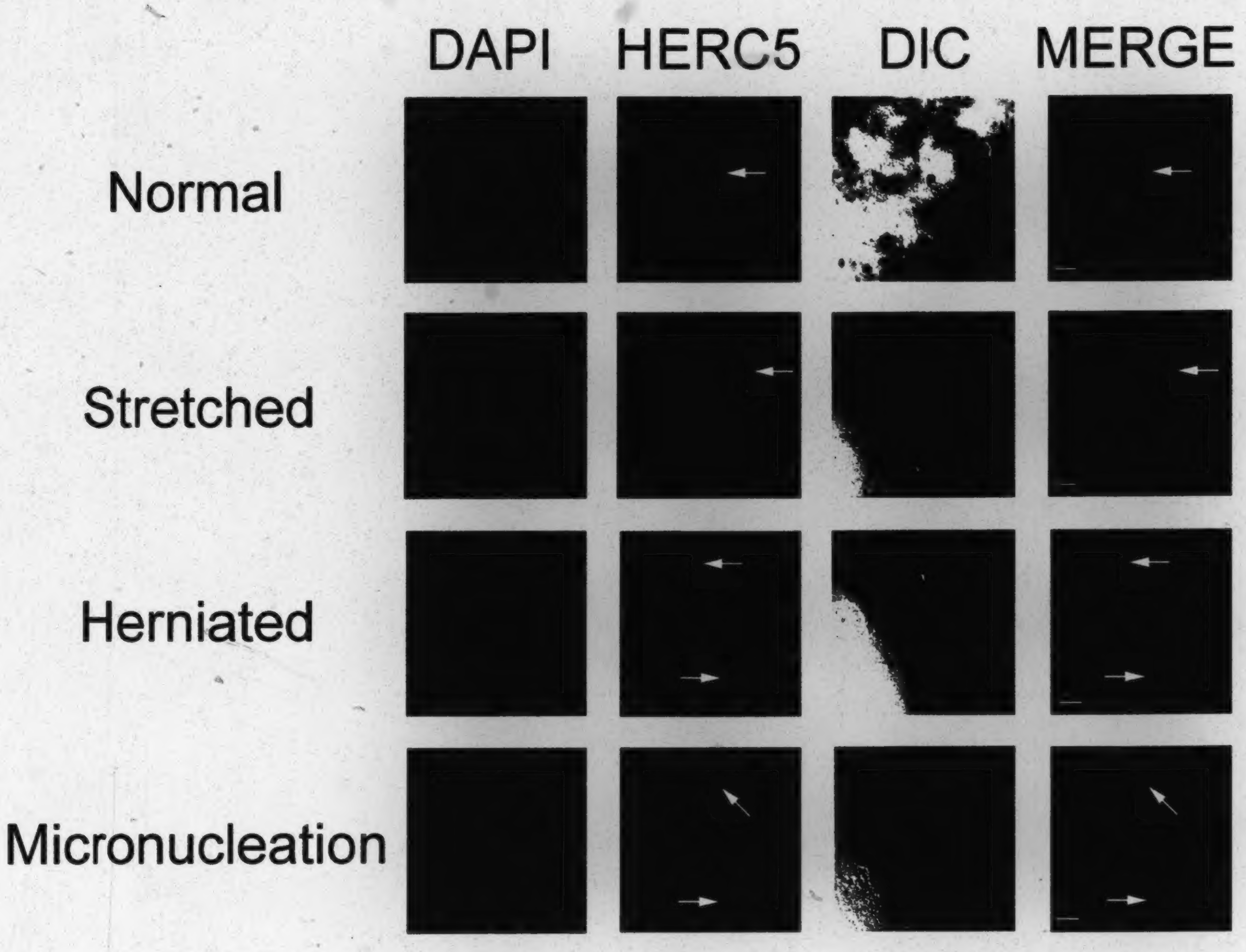

DAPI
HERC5
DIC
MERGE
Normal
Stretched
Herniated
Micronucleation

Table 3.3.2: Variations in the Nuclear Dysmorphic Phenotype of U-2 OS Cells Following Transient Transfection of pGQ06.

Variations in the occurrence of nuclear dysmorphic phenotypes following transient transfection of pGQ06. The proportion of transfected cells demonstrating nuclear dysmorphisms, and the severity of dysmorphisms, was quantitated in four separate transfections of pGQ06. HERC5 expression was detected as noted above.

Table 3.3.2. Variations in the Occurrence of Nuclear Dysmorphic Phenotypes Following Transfection of U-2 OS Cells with pGQ06 Using FuGENE6.

COVERSLIP	CELLS COUNTED	PERCENTAGE OF CELLS BEARING NUCLEAR PHENOTYPE		PERCENTAGE OF CELLS BEARING NUCLEAR PHENOTYPE, BY DYSMORPHISM		
		Normal	Dysmorphic	Stretched	Herniated	Micronucleated
pEGFP-C1	379 (Untransfected)	97.1	2.9	0.26	2.4	0.26
pEGFP-C1	600 (Transfected)	99.2	0.8	0.5	0.3	0.0
pGQ06	283 (Untransfected)	93.6	6.4	1.8	3.5	1.1
#1: pGQ06	356 (Transfected)	47.5	52.5	11.8	34.8	5.90
#2: pGQ06	394 (Transfected)	46.2	53.8	14.5	27.7	11.7
#3: pGQ06	290 (Transfected)	47.6	52.4	13.8	30.7	7.93
#4: pGQ06	384 (Transfected)	56.0	44.0	7.81	30.2	5.99
Average		49.3	50.7	12.0	30.8	7.87
Standard Deviation		4.50	4.50	2.99	2.97	2.70

Figure 3.3.3: The Nuclear Dysmorphisms in U-2 OS Cells are not Concomitant to Fragmentation of Nuclear DNA.

To determine whether the nuclear dysmorphisms seen after transient transfection of HERC5-expression plasmids were related to apoptosis, transfected cells were analysed for nuclear fragmentation. Cells were transfected with pGQ06 using FuGENE6. As a positive control for apoptosis, cells were subjected to 500µJ/cm^{2} of ultraviolet radiation 19 hours after transfection. Twenty-six hours after transfection, cells were fixed and permeablized. Ectopic expression of FLAG-HERC5 was detected using affinity-purified rabbit polyclonal antibodies to the FLAG epitope and ALEXA 546-conjugated secondary antibodies. Nuclear fragmentation was detected using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling, with FITC-dUTP as the fluorescent label and DAPI as a nuclear counterstain. Differential interference contrast (DIC) images are included for reference. Scale bars in the merged image are 10µm.

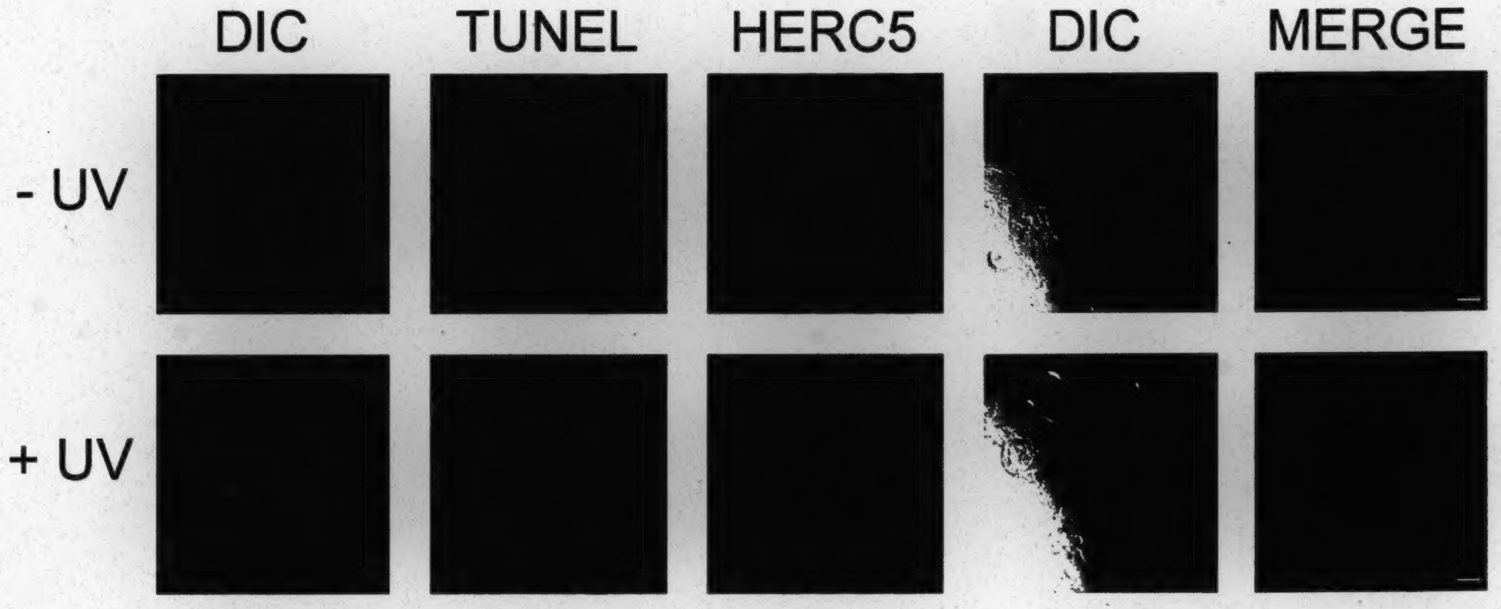

DIC
TUNEL
HERC5
DIC
MERGE
- UV
+ UV

Figure 3.3.4: Neither the RCC1-Like Domain nor HECT domain of HERC5 is Necessary for the HERC5-Dependent Nuclear Dysmorphisms in U-2 OS Cells.

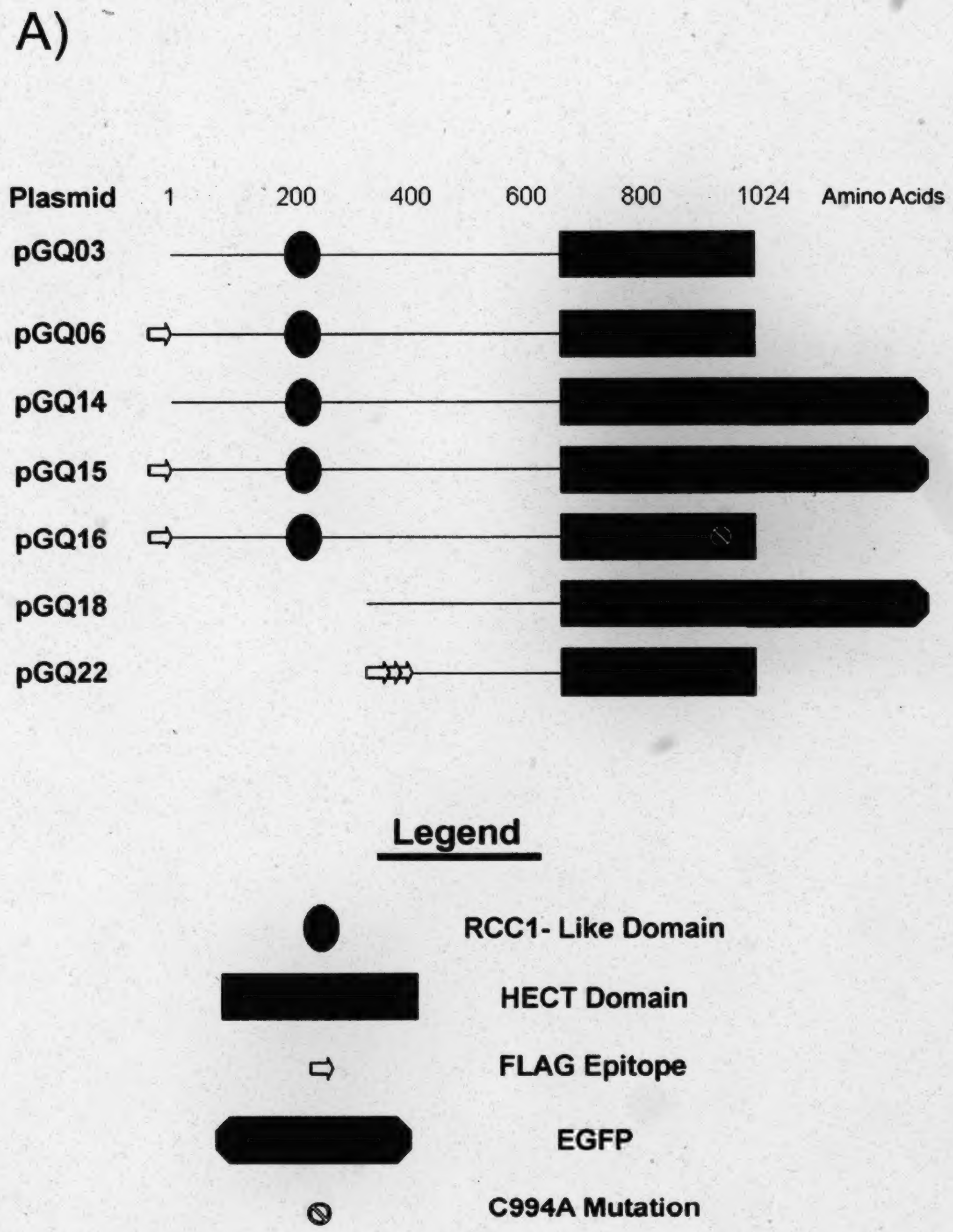
To determine which region(s) of HERC5 is involved in the nuclear deformations seen following transfection with HERC5, a panel of plasmids encoding HERC5 (bearing either a carboxy-terminal EGFP tag, amino-terminal FLAG epitope tag, or both tags) mutants (Section 2.1.7) were created. These plasmids were transfected into U-2 OS cells, and subsequently fixed and permeablized 20 hours later. Fluorescent localization of ectopically-expressed HERC5 was performed using affinity-purified rabbit polyclonal antibodies to the FLAG epitope and ALEXA 546-conjugated secondary antibodies, or by excitation of the enhanced-GFP tag, with DAPI used as a nuclear counterstain. Scale bars in the merged image are 10μm.

A) Diagram of the the protein products of the HERC5 expression vectors used.

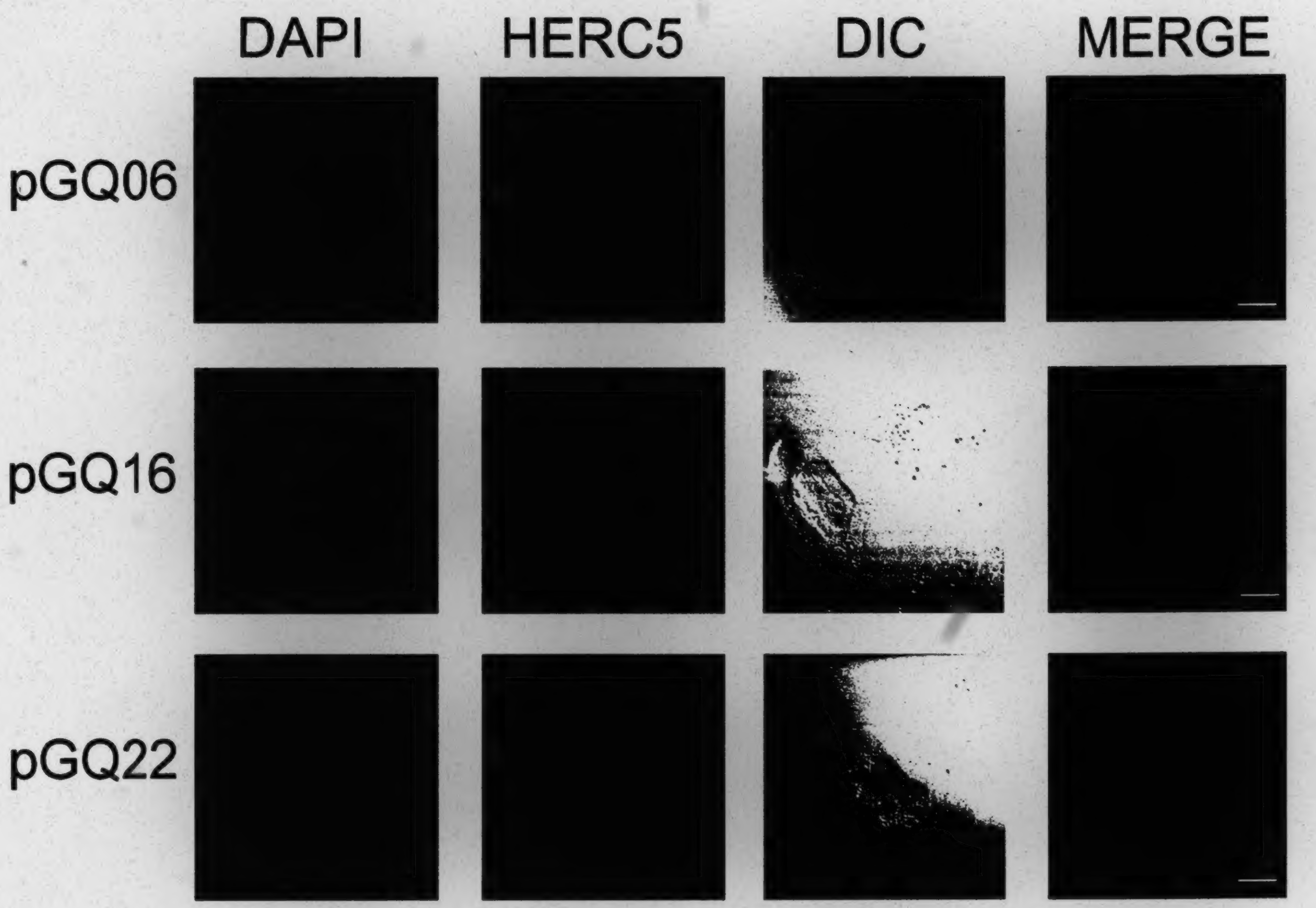
B) Images of cells transfected with the indicated plasmids, with the encoded protein product detected by anti-FLAG immunostaining.

C) U-2 OS cells transfected with the indicated plasmids, fixed and permeablized. Protein products were detected by argon laser excitation of the HERC5: enhanced-GFP fusion proteins.

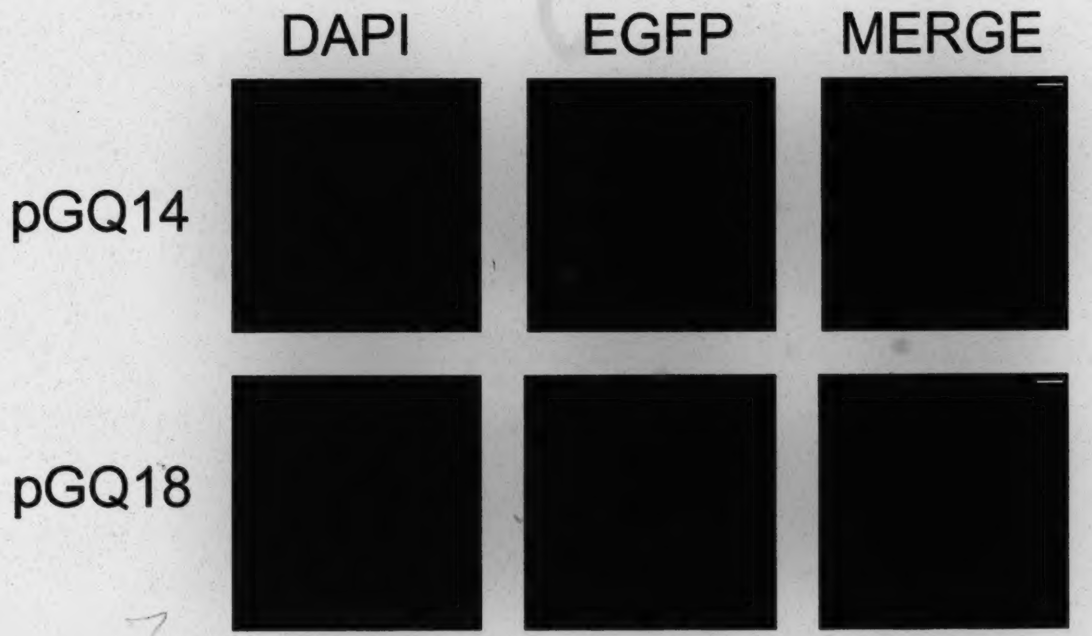
D) Image of the colocalization of both FLAG epitope tag and the EGFP domain in U-2 OS cells transfected by pGQ15. The FLAG epitope and enhanced GFP domain were detected as previously specified.


A)
Plasmid
1
200
400
600
800
1024
Amino Acids
pGQ03
pGQ06
pGQ14
pGQ15
pGQ16
pGQ18
pGQ22
Legend
RCC1- Like Domain
HECT Domain
FLAG Epitope
EGFP
C994A Mutation

B)


DAPI
HERC5
DIC
MERGE
pGQ06
pGQ16
pGQ22

C)


DAPI
EGFP
MERGE
pGQ14
pGQ18

D)

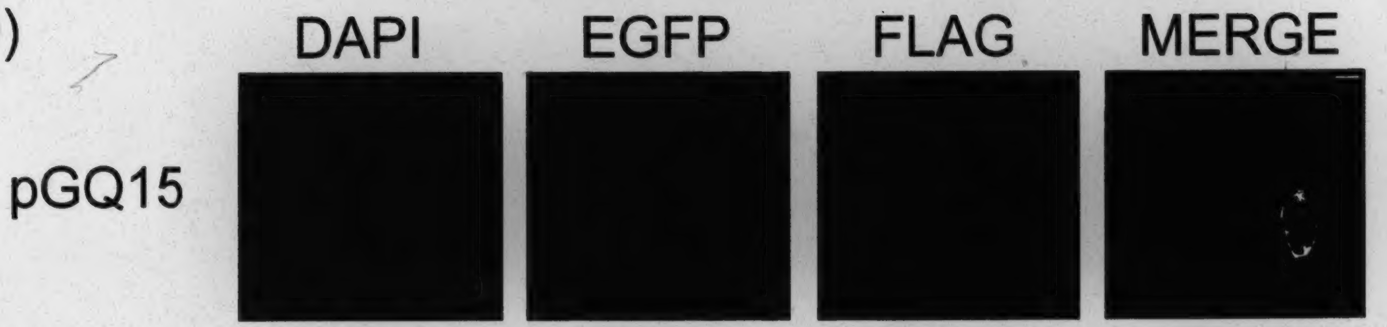

DAPI
EGFP
FLAG
MERGE
pGQ15

Table 3.3.4: Differential Abilitues of Human HERC5 Mutants to Induce Nuclear Dysmorphisms in U-2 OS Cells.

Quantitation of the nuclear morphologies demonstrated in cells transfected with various HERC5 expression vectors. Cells on a minimum of two coverslips were counted, with the nuclear morphologies categorized as illustrated in Figure 3.3.2 B.

Table 3.3.4. Differential Abilities of Human HERC5 Mutants to Induce Nuclear Dysmorphisms in U-2 OS Cells.

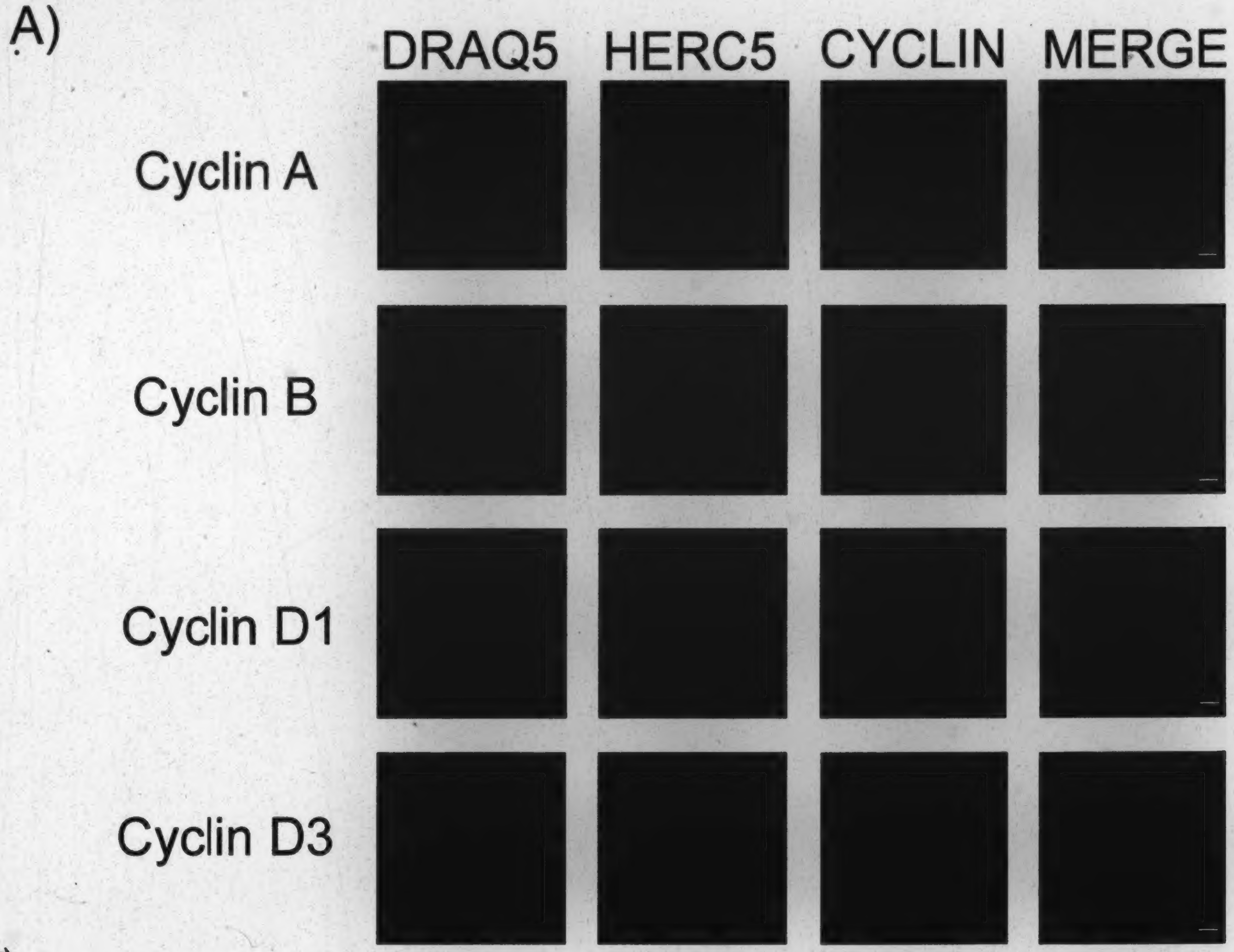
PLASMID	TRANSFECTED CELLS COUNTED	PERCENTAGE OF CELLS BEARING NUCLEAR PHENOTYPE		PERCENTAGE OF CELLS BEARING NUCLEAR PHENOTYPE, BY DYSMORPHISM		
		Normal	Dysmorphic	Stretched	Herniated	Micronucleated
pEGFP-C1	600	99.2	0.8	0.5	0.3	0.0
pGQ06	1424	49.4	50.6	11.9	30.8	9.3
pGQ14	132	54.5	45.5	0.8	41.7	3.0
pGQ15	208	68.3	31.7	0.0	28.8	2.9
pGQ16	235	57.5	42.5	0.0	40.4	2.1
pGQ18	442	53.9	46.1	2.0	30.5	13.6
pGQ22	244	82.4	17.6	0.0	11.9	5.7

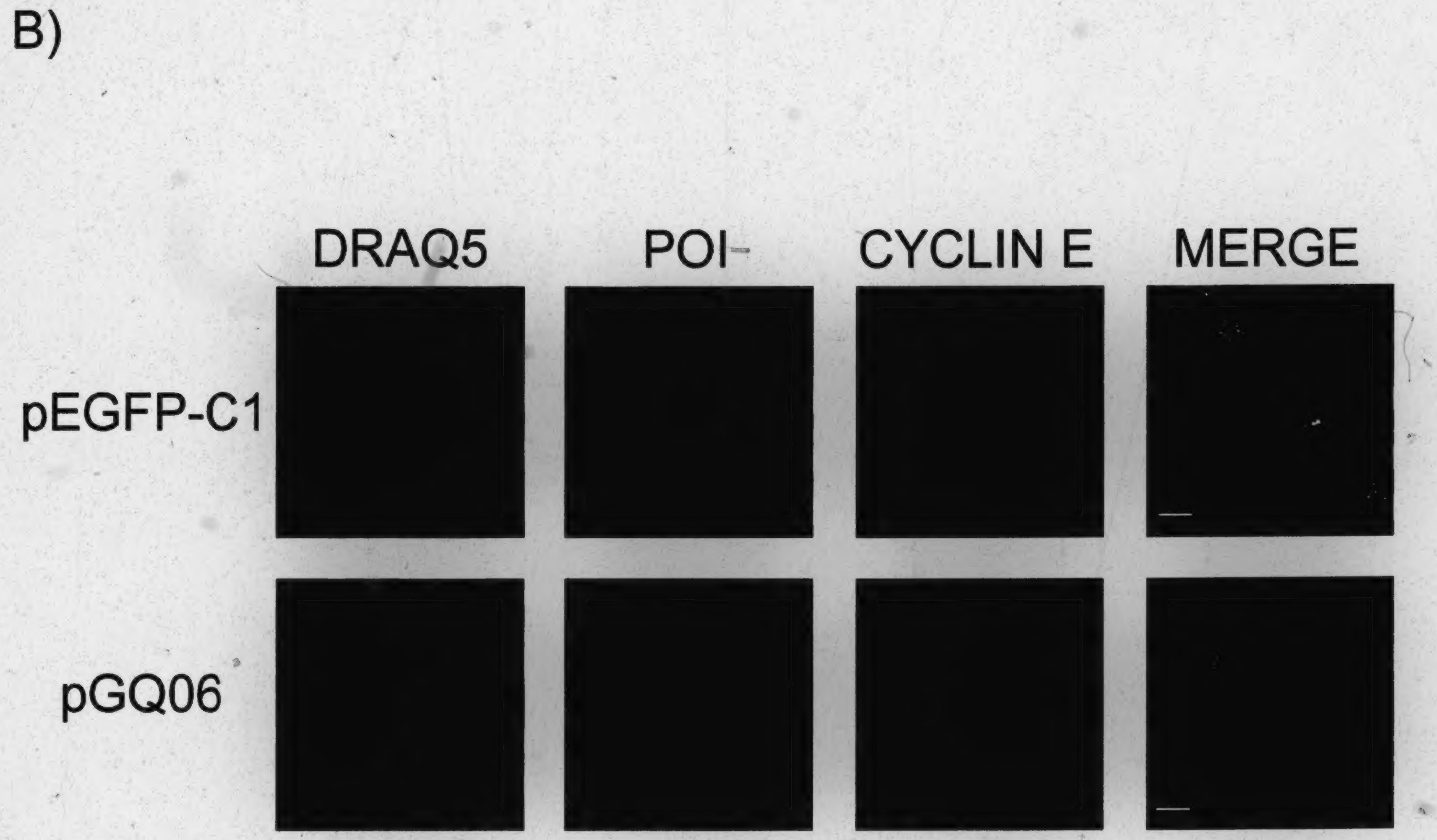
Figure 3.3.5: HERC5 Does Not Block Nuclear Cyclin Import.

A) To determine whether HERC5 may function to block nuclear import of cyclins, U-2 OS cells were transfected with pGQ06 using FuGENE6. Eighteen to twenty-four hours post-transfection, cells were fixed, permeablized, and immunostained using either mouse monoclonal (M2) or rabbit polyclonal anti-FLAG antibodies, as well as those to the cyclin of interest (noted in the left hand column). Nuclei were counterstained using DRAQ5. Scale bars in the merged image are 10μm.

B) Comparison of cyclin E localization in U-2 OS cells transfected with either pEGFP-C1 or pGQ06. To control for non-specific effects of transfection, cells were transfected with either pEGFP-C1 or pGQ06. Cells were treated identically, with the exception that the expressed enhanced GFP was detected by fluorescence excitation as opposed to immunostaining as for the FLAG epitope. Scale bars in the merged image are 10μm.

C) In order to increase cellular cyclin concentrations and detect more subtle localization effects, U-2 OS cells were transfected with either pGQ13 alone or co-transfected with pGQ06, as above. FLAG-tagged HERC5 was detected using rabbit polyclonal anti-FLAG antibodies, while cyclin E was detected using a monoclonal antibody (HE12). Nuclei were counterstained using DRAQ5. Scale bars in the merged image are 10μm.


A)
DRAQ5
HERC5
CYCLIN
MERGE
Cyclin A
Cyclin B
Cyclin D1
Cyclin D3


B)
DRAQ5
POI-
CYCLIN E
MERGE
pEGFP-C1
pGQ06

C)

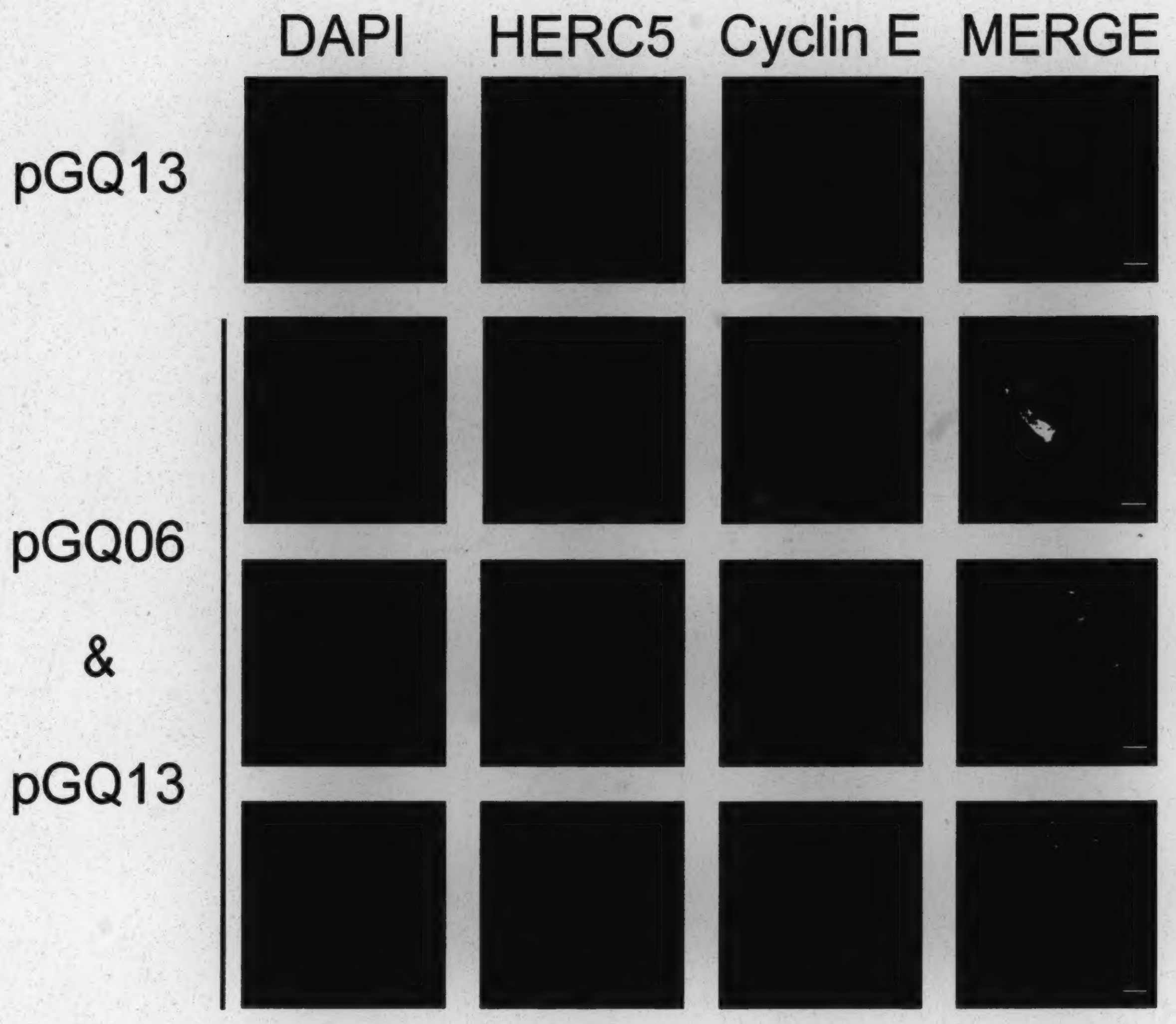
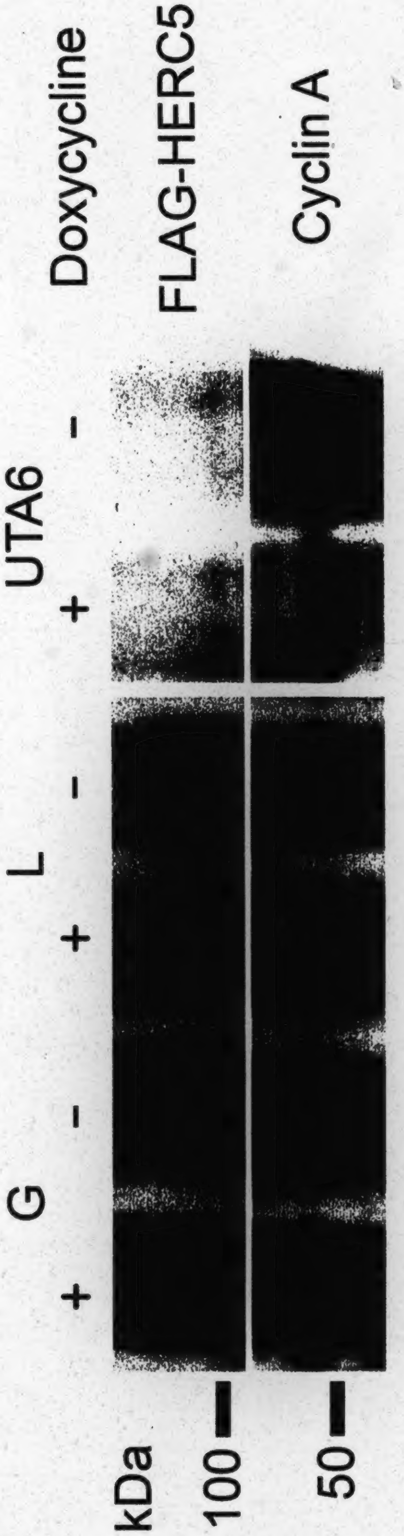

DAPI
HERC5
Cyclin E
MERGE
pGQ13
pGQ06
&
pGQ13

Figure 3.4.1: HERC5 Does Not Appreciably Affect Cyclin A Levels.

Two stably transfected clones (G and L) containing pGQ08 , a mammalian vector encoding FLAG-tagged HERC5 downstream of a tetracycline-responsive promoter, as well as the parental cell line, UTA6, were grown in either media containing or lacking doxycycline (repressive or derepressive, respectively) for 30 hours. Expression of cyclin A and FLAG-tagged HERC5 were assessed by western blotting.


Doxycycline
FLAG-HERC5
Cyclin A
G
L
UTA6
+
–
+
–
+
–
kDa
100
50

Chapter 4: Discussion

4.1 The Regulation of Human HERC5

In this work, a revised HERC5 cDNA sequence is reported, with differences to both previously reported sequences but complementary to the sequences predicted from the Celera and open human genome projects. It remains to be seen whether the previously reported differences in HERC5 cDNA sequence reflect sequencing error, novel nucleotide polymorphisms, or are products of post-translational mRNA editing in specific cell types. Further to this, it remains to be identified what effects these potential polymorphisms would have on HERC5 protein interactions and function.

Through the course of sequencing the HERC5 cDNA, an interesting repetitive motif was identified in the 3' untranslated region. The repetition of GTT motif occurs in the 3' untranslated region of several mRNAs in humans, though such sequences have no known function. It is interesting to speculate that such repeats may function to regulate mRNA stability, similar to the AU-rich instability elements, or may function to recruit translation factors. Alternatively, such repeats may be remnants of the gene duplication and translocation. The identification of proteins which might interact with such "GTT" repeats or studies into the effect of 3' untranslated region "GTT" repeat elements may have on the differential stability and/or translation of mRNAs may resolve these possibilities.

This report provides the first direct evidence that human HERC5 is regulated in response to interferon α, and is in fact a primary interferon α-inducible gene. *In silico* examination of the HERC5 promoter identifies not only STAT transcription factor elements, but also binding sites for IRF-family transcription factors (Figure 4.1). It is interesting to note that interferon γ did not induce HERC5 to detectable levels, suggesting that ISGF3 and not STAT1 homodimers are the transcriptional factors involved in HERC5 transactivation.

Additionally, it has been shown in this report that poly-inosinic: poly-cytidylic double-stranded ribonucleic acid is also capable of inducing HERC5 transcription. Based solely on the temporal activation of HERC5 in response to poly-inosinic: poly-cytidylic double-stranded ribonucleic acid, the induction of HERC5 by double stranded RNA does not appear to be a primary temporal event. When applied in the culture medium poly-inosinic: poly-cytidylic double-stranded ribonucleic acid is assumed to signal through Toll-like receptor 3, and the subsequent signal transduction events leading to the activation of IRF3 occur independent of translation. While the localization of TLR3 in human embryonic lung fibroblasts has not yet been described, though its transcript is present (personal communications with Dr. Karen Mossman), it has been described to localize to endocytic vesicles in other cell types [247, 352]. As such, TLR3 is unique amongst double-stranded RNA-responsive proteins in that it appears to sense double-standed RNA external to the cytoplasm, whereas PKR and DExH RNA helicases are thought to recognize double-stranded RNA as an intracellular pathogen-associated molecular pattern via interactions in the cytoplasm of a cell [86, 352]. While it is possible that the poly-inosinic: poly-cytidylic double-stranded ribonucleic acid is internalized by cells in endocytic vesicles and activates TLR3 incorporated into such vesicles (as opposed to TLR3 localized to the plasma membrane), it seems counterintuitive that cells would specifically uptake exogenous RNA and allow its import to the cytoplasm. Nevertheless, it remains a possibility that the poly-inosinic: poly-cytidylic double-stranded ribonucleic acid inoculated into the tissue culture medium may stimulate double-stranded RNA recognition pathways other than TLR3, including those thought to be restricted to the cytoplasm.

While the delay in the activation of HERC5 may be solely due to the kinetics of IRF3 activation, this finding may alternatively indicate that the transcription factors directly activated by TLR3 (*e.g.*, IRF3 and NFκB) alone, at least in human embryonic lung fibroblasts, are insufficient to stimulate HERC5 transcription. As IRF3 and NFκB activation are sufficient and thought to be necessary for activation of interferon β transcription, the activation of other transcription factors (including, perhaps IRF7 or ISGF3) may be required. Nevertheless, activation of cellular antiviral pathways by infection with enveloped viruses [64, 189, 259, 376] appear to quickly induce an antiviral response sufficient to induce the transcription of HERC5 and other primary interferon-stimulated genes. Further investigations into the signal transduction pathways involved in these responses, and their differences, will be of interest in elucidating how these stimuli induce HERC5 transcription. Further, the induction of HERC5 in response to poly-inosinic: poly-cytidylic double-stranded ribonucleic acid appears many fold stronger than that seen with interferon α, implying that unique factors induced as a consequence of poly-inosinic: poly-cytidylic double-stranded ribonucleic acid are required for maximal activation of HERC5. The identification of such factors will be of great interest.

Though this report identifies a novel mechanism by which HERC5 mRNA may be induced, it does little to clarify or identify the theme(s) underlying HERC5 regulation. Previous reports have suggested that HERC5 is a pro-inflammatory-inducible gene in at least a subset of endothelial cells, though not inducible by the same stimuli in human foreskin fibroblasts [194]. Additionally, HERC5 appears to be induced as a part of the antiviral response in human embryonic lung fibroblasts and several cell lines [189, 259, 376], as well as in response to interferon α and poly-inosinic: poly-cytidylic double-stranded ribonucleic acid. While detectibly expressed in cardiac tissue, HERC5 is apparently constitutively expressed nearly 100 times higher levels in testicular tissues [194, 253]. As spermatogenesis is an ongoing meiotic process occurring in germ cells in the testes, these cells may be expected to undergo many processes reported to induce HERC5 expression: telomerase activity [201, 327], have deactivated p53 and pRB as a consequence of meiotic progression [253], and potentially have increased levels of c-myc to promote continued cell cycling [250]. The induction of HERC5 in response to interferon α and the antiviral response, a process known to induce cell cycle arrest, as well as in response to deregulation of cell cycle factors, which leads to deregulated cell cycle progression, appear paradoxical.

One hypothesis which may reconcile these observations is that HERC5 may act as a checkpoint for checkpoint regulators, an "emergency brake" transcriptionally activated in response to loss or inactivation of cell cycle checkpoints, or in the case of activation of an antiviral or interferon response, to ensure that these checkpoints are maintained to prevent viral manipulation. Though no evidence directly associates HERC5 with cell cycle modulation, these patterns of regulation, especially combined with its reported ability to bind cyclins, are strongly suggestive that it functions in this area. Examination of the effects of HERC5 on cell cycle progression, as well as further characterization of its protein interactions, especially those with cell cycle regulatory proteins, may elucidate if HERC5 actually does function in cell cycle regulation.

4.2 HERC5-Induced Nuclear Dysmorphisms

Two previous studies have identified the localization of HERC, with similar results obtained in several cell types [143, 253]. In these studies, as in this report, HERC5 appears to be completely excluded from the nucleus. Unlike the previous studies, HERC5 localization did not appear punctuate or to have a perinuclear concentration [143, 253]. Whether this reflects an artefactual variation due to fixation and permeablization methods or immunological reagents remains undefined. Alternatively, this may represent a real difference in the cell types used. Unique to this report, ectopic expression of HERC5 has been shown to induce nuclear dysmorphisms in U-2 OS osteosarcoma cells. This phenomenon did not appear occur in 293T transformed human embryonic kidney cells or A549 lung carcinoma cells, suggesting that it may occur as a combined consequence of HERC5 expression and other events inherent to the transformed nature of U-2 OS cells.

While the mechanism and molecular consequences of the transformation of U-2 OS cells are not well defined, several unique details are known. Unusual for a highly transformed cell type, U-2 OS cells express function pRB and p53, the expression of which has been reported to suppress HERC5 transcription [253]. However, the promoters of p16^{INK4A} and p14^{ARF} (tumour suppressor proteins which promote the function of pRB and p53 respectively) are reportedly methylated in U-2 OS cells, thereby preventing their expression [290]. Ectopic expression of either p16^{INK4A} and p14^{ARF} suppressed the growth of U-2 OS cells, suggesting that the silencing of these genes is critical to the transformation of U-2 OS cells [290]. Attributable as a consequence of the functional pRB and p53, U-2 OS cells were reported not to constitutively express HERC5 mRNA, unlike most transformed cell lines [253]. However, the finding that HERC5 is not induced by interferon α in U-2 OS cells suggests a further defect in this cell type, potentially loss of the HERC5 gene. Spectral karyotypings studies of the genetic rearrangements in U-2 OS cells suggest that the region of chromosome 4 encoding HERC5 (4q21-22) is intact [17]. Despite this, karotyping does not provide resolution sufficient to identify whether the HERC5 gene and its promoter remain intact and capable of transactivation. Additionally, epigenetic silencing of the HERC5 locus, or defects in components of the pathways involved in the activation of the HERC5 promoter remain alternative possible explanations for the loss of HERC5 expression. Reports that U-2 OS cells have defects in the localization of hMPS1 appear to be based on artefactual differences attributed to immunological reagents used by different groups [377]. Further identification of the molecular events resulting in the transformation of these cells will be of potential interest in identifying the cause of transcriptional silencing of HERC5.

As U-2 OS cells ectopically expressing HERC5 are negative for TUNEL staining, implying that the nuclear dysmorphic cells are not undergoing apoptosis (or at least, have not progressed to the point at which nucleolytic enzymes are activated), it is difficult to categorize the events occurring. Though the nuclear dysmorphic phenotypes illustrated in this report bear striking similarity to those described in various reports of mitotic catastrophe, it remains to be shown that the affected proportion of U-2 OS cells have entered mitosis. Indeed, the DIC images of nuclear dysmorphic cells appear to have a well-defined nuclear boundary as well as defined boundaries for micronuclei, implying that dissolution of the nuclear envelope(s) has not occurred. Alternatively, it is possible that the cells are indeed mitotic but a function of HERC5 effectively inhibits nuclear membrane dissolution or actively enhances nuclear membrane reformation [18, 242, 336].

As the molecular events associated with mitotic catastrophe remain uncharacterized, it remains vague what could or should not be associated with this type of cell death. While it seems highly unlikely that cells bearing the more severe forms of nuclear dysmorphism could remain viable for subsequent mitotic cycles, there is no direct evidence that these events are part of a cell death pathway, nor evidence that these events are directly associated to cell cycle factors. Rather, these nuclear dysmorphic events may be simply reflective of inappropriate modulation of the cytoskeleton – potentially including premature activation of spindle microtubule formation [283]. As nuclear lobulation is a normal and expected feature of polymorphonuclear leukocytes, which are terminally differentiated cells, it naturally follows that such alterations in nuclear structure (sometimes indistinct from those seen following ectopic expression of HERC5 in U-2 OS cells) need not follow as a consequence of mitotic processes. Rather, it is possible that dysregulation of cytoskeletal regulatory factors in any phase of the cell cycle might lead to abnormal nuclear morphologies or bypass of cytoskeletal checkpoints ensuring maintenance of form [267]. Nevertheless, aberrant modulation of critical cell cycle factors is clearly able to induce highly similar dysmorphisms [43, 49, 52, 58, 62, 96, 107, 152, 240, 271, 274, 353, 354, 356, 368, 381, 395, 401, 407, 421, 437, 447], and therefore such possibilities should be examined further.

The use of expression vectors encoding modified forms of HERC5, bearing deletions or point mutations affecting the HERC5 primary structure and presumably also the function, suggests that neither the RCC1-like nor HECT domains are required for the nuclear dysmorphisms seen. It remains possible that inter-domain region of HERC5 (putatively involved in cyclin-binding [253]) bears some currently-unrecognized enzymatic function, though seems more likely that protein interactions mediated by this regions are responsible for this effect. As transfection with other expression vectors did not induce such nuclear dysmorphisms, it appears unlikely that these events are a non-specific artefact attributable to transfection or ectopic over-expression of a protein. It is possible, however, that unusual genetic events such as site-specific recombination or production of nucleic acids which activate RNA interference events may alternatively be responsible, though unlikely as the nuclear dysmorphic phenotype appears (of the cell lines studied) to affect U-2 OS cells alone.

In order to resolve the mechanism by which ectopic expression of HERC5 induces nuclear dysmorphisms, further study of the protein interactions of HERC5 is essential. Identification of proteins which interact with HERC5, especially those which interact within the inter-domain (and putatively cyclin-binding) region of HERC5 will provide a basis for further studying HERC5 function and the mechanism of nuclear dysmorphic events in U-2 OS cells. Following identification of HERC5 binding partners, abrogating such interactions by point mutations/deletions will provide a direct screen to determine which protein interactions are required for the induction of nuclear dysmorphisms, and if indeed modulation of either the cytoskeleton and/or cell cycle factors are involved.

4.3 HERC5 and Its Effects on Cyclins

In its initial characterization, HERC5 was found to interact with cyclins A1, B1, D1, and E [253]. As most cyclins, with the exception of cyclins B and G2, are quickly imported to the nucleus following translation, where it is thought their primary functional activity occurs, the exclusively cytoplasmic localization of HERC5 brings to question how HERC5 affects cyclin localization. Strangely, no further study of HERC5-cyclin interactions was pursued past the identification of their interaction (by yeast two-hybrid, immunoprecipitation, and GST pulldown techniques [253]). Based on the ability of HERC5 to bind cyclins and the suggested cell cycle regulatory function of HERC5, it could be speculated that HERC5 may prevent nuclear import of cyclins.

Examination of the localizations of cyclins and ectopically expressed HERC5 by confocal immunofluorescence suggests that HERC5 does not appreciably affect the localization of the endogenous cyclins studied, save for cyclin E. When both cyclin E and HERC5 were ectopically expressed, a small proportion of cyclin E appeared to be retained in the cytoplasm, dependent on the co-expression of HERC5. It remains to be seen whether the retention of cyclin E in the cytoplasm is a direct effect due to interaction with HERC5, or whether the retention is an indirect effect mediated through the interaction of HERC5 (and possible enzymatic function) with other interacting proteins. It is quite possible that HERC5 could associate with other factors to effect cell cycle arrest in G1 phase, inducing an inhibition of cyclin E-CDK2 activation and leading to the increased cytoplasmic localization of cyclin E.

Nevertheless, with a previous report demonstrating that HERC5 does interact with cyclin E [253] and the altered localization pattern noted in this study, it seems probably that HERC5 can functionally interact with the cyclin E. However, it remains to be demonstrated that this interaction is physiologically relevant. Indeed, in the initial characterization of HERC5, the cyclin interaction was only demonstrated by ectopic co-expression [253]. Thus, it is possible that the interaction of HERC5 with these cyclins is an artefact of over-expression, *i.e.*, it may require excessive amounts of HERC5 to either out saturate physiological ligands or to reach a great enough concentration to push the equilibrium to bias the association of these HERC5 with cyclin E. Alternatively, the interaction of HERC5 with cyclins may be highly transient, though this possibility is discouraged by the co-immunoprecipitation and GST pulldown of cyclins with HERC5 [253].

In order to elucidate whether cyclins naturally interact with HERC5, co-immunoprecipitation of HERC5 with endogenous binding partners following transcriptional induction of HERC5 (potentially by interferon α or poly-inosinic: poly-cytidylic double-stranded ribonucleic acid), followed by identification of interacting partners by mass spectrometry would be of immense value. Presuming that the interactions of HERC5 with cyclins are physiological, it remains possible that a cyclin other than those studied here is a natural binding partner. While the cyclins examined here, with the exception of cyclin B1, are predominantly nuclear-localized, several other cyclins appear to be predominantly cytoplasmic. These cyclins, including cyclins B1, B2, and G2, seem likely to have extra-nuclear functions. Cyclin B1 has many well characterized functions in promoting exit from G2 phase and entry into mitosis, stimulating events such as chromosome condensation, dissolution of the nuclear lamina and envelope, formation of the mitotic spindle, and cytoskeletal rearrangements inherent in mitotic progression. Cyclin B1 is retained in the cytoplasm until entry into prophase [303]. While in the cytoplasm, cyclin B1 appears to localize with microtubules, including those of the mitotic spindle when present [10, 162, 238, 284]. In contrast, cyclin B2 appears to be largely associated with the Golgi apparatus and does not undergo nuclear translocation during mitosis [162]. Cyclin B2 appears to play key distinct functions in disassembly of the Golgi apparatus during mitosis [89], as well as in proper formation of bipolar mitotic spindles [191]. Unlike cyclin B1 knockout mice, which die *in utero*, cyclin B2 knockout mice are viable, fertile, and display no obvious abnormalities [35]. While cyclin B2 is highly expressed during spermatogenesis and oogenesis, as well as in lymphoid organs, no apparent abnormalities in cell growth or replication were noted in these tissues [35], perhaps suggesting that cyclin B1 may be able to compensate in the absence of cyclin B2.

Though cyclin G1 and cyclin G2 appear highly similar, their regulation and functions appear distinct. While cyclin G1 is predominantly nuclear localized and is a p53-responsive gene [150, 282], cyclin G2 is primarily cytoplasmic and does not appear to be regulated in response to p53 [19]. Rather, cyclin G2 demonstrates strong expression in the cerebellum, kidney, and lymphoid tissues, but appears to be strongly expressed in apoptotic cells [149, 150, 282]. When ectopically expressed in HEK 293 (adenoviral E1A, E1B transformed human embryonic kidney cells), cyclin G2 expression resulted in nuclear dysmorphisms similar to those noted following ectopic HERC5 expression in U-2 OS cells [19]. It is interesting to note that HEK 293 cells appear to constitutively express HERC5 mRNA and protein, while U-2 OS cells appear unable to express HERC5 (results herein and [253]). While the expression of cyclin G2 in U-2 OS has yet to be investigated, it is tempting to speculate that the nuclear events result from an activity promoted by HERC5-cyclin G2 interaction.

Cyclins with significant cytoplasmic localization appear much more likely to act as HERC5 binding partners, based on the confocal cyclin-HERC5 colocalization study presented herein. While many of the recently identified cyclins, including cyclins F, G1, G2, and I, remain poorly characterized, what little is known suggests that these proteins play powerful roles in cell function, similar to their better-characterized family members [188, 314, 317, 405]. Further study of these cyclins and their potential interaction with HERC5 may provide a clear link to the nuclear dysmorphic effects noted in U-2 OS cells.

4.4 Future Directions

Through the attempt to further characterization of HERC5, this report has identified several unique events and raised more questions than it has provided definitive answers. While evidence presented herein clearly demonstrate that HERC5 is a novel primary interferon α-stimulated gene, which is also apparently responsive to extracellular double-stranded RNA, nothing is known of what role HERC5 may play in these responses. Just as importantly, it remains to be shown whether HERC5 exerts an antiviral function at all, though preliminary data suggests that its ectopic expression in fact does inhibit the replication of wild-type HHV1 (personal communications with Meaghan Hancock). While modulation of the cell cycle, one widely postulated function of HERC5, would likely have an antiviral effect on at least nuclear DNA viruses [343], modulation of cytoskeletal functions or vesicle trafficking functions for HERC5 may elicit similar antiviral functions (with expected resistance to a broader range of pathogens). Demonstration of HERC5 antiviral function, with differential analysis of the effect of HERC5 expression on different families and types of viruses, may provide key insights into HERC5 function and allow determination of the domains required for such effects. Additional characterization of the point(s) HERC5 expression affects the viral life cycle may aid elucidation of HERC5 function(s).

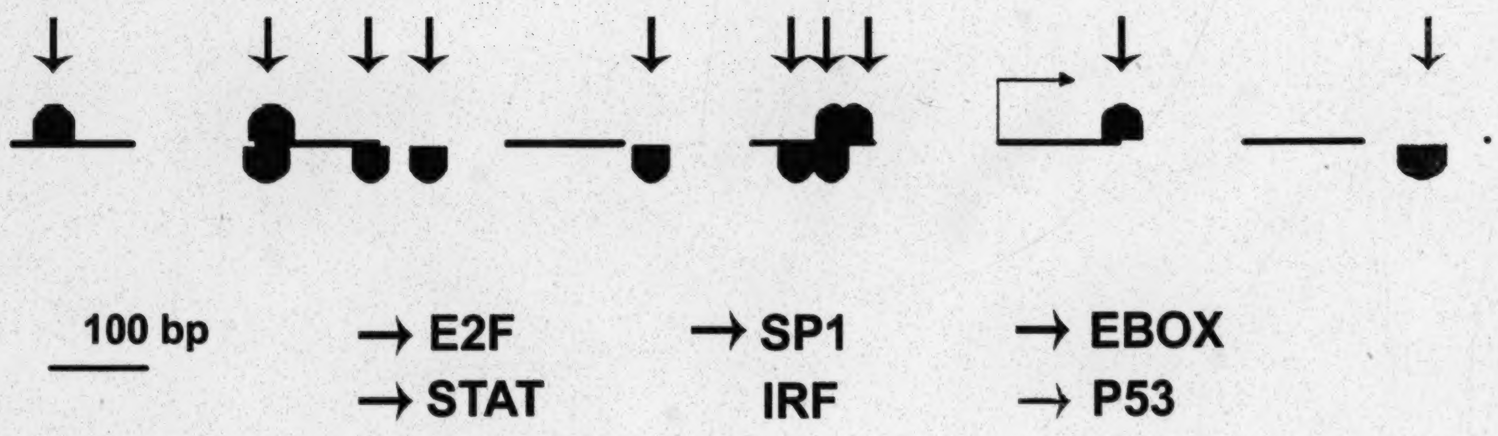
Save for its reported interactions with cyclins [253] and UbcH5a [194], nothing is known of HERC5 interactions with other proteins. Further identification of interacting partners may aid in the identification of the functions of the HERC5

RCC1-like and HECT domains. It would be of great interest to determine if HERC5 functions as a ubiquitin E3 ligase, and if so, whether it forms conventional K48-linked polyubiquitin chains or rather alternatively linked chains, suggestive of a signalling role for such labelled proteins. As alternatively suggested, HERC5 may act as an E3 ligase for ISG15 [439]. Should this be the case, it would be the first reported case of a HECT domain ligase acting as an E3 enzyme for a ubiquitin-like protein. Additionally, further investigation into interacting partners and protein function may elucidate the functional relevance of the domain architecture of HERC family proteins.

The normal tissue distribution of HERC5 mRNA suggests minimal transcription in most tissues but strong expression of HERC5 in the testes and a weaker expression in ovarian tissue [143, 253]. While the function(s) of HERC5 in these tissues remain unknown, it is interesting to note that ovarian and testicular tissues contain germ cells, and meiosis occurs in these tissues. It is attractive to speculate that HERC5 may play an additional role in meiosis or in maintenance of germ cells. It is a puzzling addition to this picture that in many somatic cell types HERC5 seems to be a gene responsive to danger signals [189, 194, 259, 376]. The seemingly unique regulation of HERC5 in microvascular endothelial cells in response to pro-inflammatory stimuli may suggest a unique role in this tissue [194], perhaps involved in the events allowing extravasation of immune cells across the endothelium. The activation of HERC5 transcription in response to viral infection and stimuli which activate and perpetuate the antiviral response is also of great interest. While nothing is currently known of the function of HERC5 in this regard, it is enticing to speculate that it performs a novel antiviral function. Indeed, with evidence that both the ISG15 and ubiquitin conjugating systems appear to be induced during an antiviral response [275], the presence of a HECT E3 ligase domain in HERC5 makes this a tempting conclusion. Clearly, much further research is needed to elucidate the cryptic function of this protein and regulation of this gene.

Figure 4.1: Bioinformatic Analysis of Transcription Factor Binding Sites in the HERC5 Promoter Region.

The sequence of HERC5 promoter region was extracted from the human genome using Genomatix Gene2Promoter (www.genomatix.de), with potential transcription factor binding sites identified using MatInspector software (www.genomatix.de) [3]. Binding sites for selected transcription factor families (as identified by MatInspector) are indicated by coloured shapes on the top (sense strand of DNA) or bottom (anti-sense strand of DNA). The translational start site is represented by a red arrow indicating the site and direction of translation.


100 bp
→ E2F
→ STAT
→ SP1
IRF
→ EBOX
→ P53

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